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**(54) Title:** STABILIZATION OF POLYNUCLEOTIDE COMPLEXES**(57) Abstract**

Polynucleotide complexes are stabilized by adding a cryoprotectant compound and lyophilizing the resulting formulation. Cryoprotectant compounds comprise carbohydrates, preferably lactose and sucrose, but also glucose, maltodextrins, mannitol, sorbitol, trehalose, and others. Betaines, prolines, and other amino acids may also be useful. Preferably, DNA complexes are cryoprotected with lactose at concentrations of about 1.25 % to about 10 % (w/vol). Conventional buffers may also be added to the mixture. The lyophilized formulations may be stored for extended periods of time and then rehydrated prior to use.

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## STABILIZATION OF POLYNUCLEOTIDE COMPLEXES

### Relation to Other Applications

5        This application is a continuation-in-part of US Application Serial No. 08/092,200 filed July 14, 1993 and US Application Serial No. 07/913,669 filed July 14, 1992, which is a continuation-in-part of US Application Serial No. 07/864,876 filed April 3, 1992, now abandoned.

10        Background Art

Molecular biologists have identified the chromosomal defects in a large number of human hereditary diseases, raising the prospects for cures using gene therapy. This emerging branch of medicine aims to correct genetic defects by transferring cloned and functionally active 15 genes into the afflicted cells. Several systems and polymers suitable for the delivery of polynucleotides are known in the art. In addition, gene therapy may be useful to deliver therapeutic genes to treat various acquired and infectious diseases, autoimmune diseases and cancer.

Despite the usefulness of polynucleotide delivery systems, such 20 systems are metastable and typically exhibit a decrease in activity when left in solution for longer than a few hours. For example, conventional cationic-lipid mediated gene transfer requires that the plasmid DNA and the cationic lipid be separately maintained and only mixed immediately prior to the gene transfer. Current attempts to stabilize polynucleotide 25 complexes comprise speed-vac or precipitation methods, but they do not maintain activity over suitable time periods. Attempts to store polynucleotides in salt solutions lead to a loss of supercoil structure. If gene therapy protocols are to become widely used it will be necessary to have a stable and reproducible system for maintaining activity. This 30 is of particular importance to pharmaceutical and commercial uses. Accordingly, there remains a need for means to stably maintain

polynucleotide compositions for extended periods of time. The present invention satisfies this and other needs.

#### Summary of the Invention

5        The invention comprises a method of stabilizing polynucleotide complexes by adding a cryoprotectant compound and lyophilizing the resulting formulation. Cryoprotectant compounds comprise carbohydrates, preferably lactose and sucrose, but also glucose, maltodextrins, mannitol, sorbitol, trehalose, and others. Betaines  
10      prolines, and other amino acids may also be useful. Preferably, the invention comprises DNA complexes cryoprotected with lactose at concentrations of about 1.25% to about 10% (w/vol). Conventional buffers may also be added to the mixture. The invention also comprises the lyophilized mixtures.

15      The lyophilized formulations may be stored for extended periods of time and then rehydrated prior to use. In an alternative embodiment, the lyophilized formulations may be milled or sieved into a dry powder formulation which may be used to deliver the polynucleotide complex. Once the powder contacts the desired tissue, it rehydrates, allowing  
20      delivery of the polynucleotide complex. In a preferred embodiment, a dry powder formulation is used to induce genetic modification of a patient's lung tissue.

#### Brief Description of the Drawings

25      FIG. 1 shows the effect of rehydration on the particle size distribution of lipid-polynucleotide complexes at varying concentrations of mannitol and lactose.

FIG. 2 compares the particle size distribution of lipid-polynucleotide complexes before lyophilization and after rehydration with  
30      and without cryoprotectant.

FIG. 3 shows the particle size distribution for various lipid-polynucleotide complexes before and after lyophilization.

FIG. 4 illustrates the effect of cryoprotectant on the zeta potential of lipid-polynucleotide complexes.

5 FIG. 5 is a graphical representation of gel electrophoresis results indicating the effect of lyophilization on complexation between lipid and polynucleotide.

FIGs. 6 and 7 show dose response curves of transfection efficiency comparing lyophilized and non-lyophilized lipid-polynucleotide  
10 complexes.

FIGs. 8-10 show the effect of lyophilization on transfection efficiency for lipid-polynucleotide complexes.

FIGs. 11-13 illustrate the effect of time on the transfection efficiency of lyophilized lipid-polynucleotide complexes.

15 FIG. 14 illustrates the effect of time on the transfection efficiency of rehydrated lipid-polynucleotide complexes.

FIG. 15 illustrates transfection using lyophilized dendrimer-polynucleotide complexes.

20 FIGs. 16-17 illustrate transfection using other lipid-polynucleotide complexes.

FIG. 18 shows expression of genetic information transferred using a dry powder formulation of a lyophilized polynucleotide complex.

FIG. 19-31 show various cationic lipids useful in forming lipid:polynucleotide complexes for lyophilization.

25 FIG. 32 shows predicted deposition sites in the respiratory tract for various size particles.

#### Detailed Description of the Drawings

The invention comprises stabilizing polynucleotide complexes by adding  
30 a cryoprotectant and lyophilizing the resulting mixture. Cryoprotectant compounds comprise carbohydrates, preferably lactose and sucrose, but

also glucose, maltodextrins, mannitol, sorbitol, trehalose, and others. It is believed the hydroxyl groups of the carbohydrates form hydrogen bonds with the polynucleotide complexes, displacing water and stabilizing the complexes. Useful ranges of cryoprotectant range from 5 about 1.25% to about 10%, and particularly from 5-10%. Other suitable cryoprotectants include amino acids such as betaines and prolines that exhibit this hydrogen bonding stabilization effect.

A wide variety of polynucleotide complexes may be stabilized with the lyophilization techniques of this invention. The polynucleotide may 10 be a single-stranded DNA or RNA, or a double-stranded DNA or DNA-RNA hybrid. Triple- or quadruple-stranded polynucleotides with therapeutic value are also contemplated to be within the scope of this invention. Examples of double-stranded DNA include structural genes, genes including operator control and termination regions, and self-15 replicating systems such as plasmid DNA, among others.

Single-stranded polynucleotides or "therapeutic strands" include antisense polynucleotides (DNA and RNA), ribozymes and triplex-forming oligonucleotides. In order to have prolonged activity, the therapeutic strand preferably has as some or all of its nucleotide linkages stabilized 20 as non-phosphodiester linkages. Such linkages include, for example, phosphorothioate, phosphorodithioate, phosphoroselenate, or O-alkyl phosphotriester linkages wherein the alkyl group is methyl or ethyl, among others.

For these single-stranded polynucleotides, it may be preferable to 25 prepare the complementary or "linker strand" to the therapeutic strand as part of the administered composition. The linker strand is usually synthesized with a phosphodiester linkage so that it is degraded after entering the cell. The "linker strand" may be a separate strand, or it may be covalently attached to or a mere extension of the therapeutic 30 strand so that the therapeutic strand essentially doubles back and hybridizes to itself. Alternatively, the linker strand may have a number

of arms that are complementary so that it hybridizes to a plurality of polynucleotide strands.

The linker strand may also have functionalities on the 3' or 5' end or on the carbohydrate or backbone of the linker that serve as functional

5 components to enhance the activity of the therapeutic strand. For example, the phosphodiester linker strand may contain a targeting ligand such as a folate derivative that permits recognition and internalization into the target cells. If the linker is attached to its complementary therapeutic strand that is composed of degradation-resistant linkages, 10 the duplex would be internalized. Once inside the cell, the linker will be degraded, thereby releasing the therapeutic strand. In this manner, the therapeutic strand will have no additional functionalities attached and its function will not be impeded by non-essential moieties. This strategy can be applied to any antisense, ribozyme or triplex-forming 15 polynucleotide and it is used to deliver anti-viral, anti-bacterial, anti-neoplastic, anti-inflammatory, anti-proliferative, anti-receptor blocking or anti-transport polynucleotides, and the like.

A separate linker strand may be synthesized to have the direct complementary sequence to the therapeutic strand and hybridize to it in a one-on-one fashion. Alternatively, the linker strand may be constructed so that the 5' region of the linker strand hybridizes to the 5' region of the therapeutic strand, and the 3' region of the linker strand hybridizes to the 3' region of the therapeutic strand to form a concatenate of the following structure.

25      5' \_\_\_\_\_  
            3' \_\_\_\_\_

This concatenate has the advantage that the apparent molecular weight of the therapeutic nucleic acids is increased and its pharmacokinetic properties are modified.

30 adjusted to achieve the optimal therapeutic effect. The linker strand may also be branched and able to hybridize to more than one copy of

the polynucleotide. Other strategies may be employed to deliver different polynucleotides concomitantly. This would allow multiple genes to be delivered as part of a single treatment regimen.

The polynucleotide complex may comprise naked polynucleotide 5 such as plasmid DNA, multiple copies of the polynucleotide or different polynucleotides, or may comprise a polynucleotide associated with a peptide, a lipid including cationic lipids, a liposome or lipidic particle, a polycation such as polylysine, a branched, three-dimensional polycation such as a dendrimer, a carbohydrate or other compounds that facilitate 10 gene transfer. Examples of useful polynucleotide compositions are found in U.S. Patent Applications Ser. No. 08/092,200, filed July 14, 1992, and Serial No. 07/913,669, filed July 14, 1993, which are hereby incorporated in their entirety by reference thereto.

15 Results

A 1:1 (w/w) liposome formulation containing the cationic lipid n-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride [DOTMA](obtained from Syntex Inc. (U.S.A.)) and dioleoyl phosphatidylethanolamine (DOPE) was prepared by rehydration of a lipid 20 film with subsequent extrusion under pressure using a 100 nm pore size polycarbonate membrane. Cryoprotectant, lipid and plasmid DNA, containing a CMV promoter and a  $\beta$ -galactosidase (CMV- gal) or chloroamphenicol acetyl transferase (CMV-CAT) reporter gene were mixed together under defined conditions to produce a 1:10:X (w:w:w) 25 of pDNA/lipid/cryoprotectant formulation at a constant pDNA concentration of 250  $\mu$ g/ml in a final volume of 1 ml, where X was 30, 100, 200, 250, 300, 500, 600, 750 and 100. This corresponds to a DNA:lipid charge ratio of 1:2. The cryoprotectants used were mannitol and lactose. The formulations were lyophilized using a programmable 30 tray dryer (FTS Systems) at a product eutectic temperature of -30°C. The lyophilized formulations were rehydrated at room temperature with

water to a pDNA concentration of 250  $\mu$ g/ml. After 30 minutes, the physicochemical properties of the lipid-pDNA complexes was determined by particle size analysis (Coulter N4MD), doppler electrophoretic light scattering (Coulter Delsa 440) and 1% agarose gel electrophoresis.

5 For *in vitro* studies, transfection efficiency of the pDNA-lipid-cryoprotectant formulations was studied on a variety of cell lines. HIG-82 (rabbit synoviocytes), C<sub>2</sub> C<sub>12</sub> (mouse myoblasts) and HepG2 (human liver hepatoblastoma) cells were grown in F-12 Ham's, Dulbecco's Modified and in minimum essential Eagle's media (Gibco), respectively.

10 All were supplemented with 10% fetal bovine serum (Gibco). Transfection was performed in the presence of serum containing media in 24-well plates at 40-60% cell density with 2  $\mu$ g of pDNA per well. Cells were harvested and analyzed after 48 hours. A chemiluminescent reporter assay was performed according to TROPIX (Galacto-light )

15 specifications. The percentage of  $\beta$ -galactosidase (LacZ) positive cells was determined by Commasie Blue protein assay. Relative light units (RLU) per  $\mu$ g of total protein and percentage of LacZ positive cells were used to assess transfection efficiency.

FIG. 1 shows the effect of rehydration on the particle size  
20 distribution of lipid-pDNA complexes at varying concentrations of mannitol and lactose. At cryoprotectant:lipid ratios of 40:1 to 100:1 (corresponding to 4% and 10% formulations), complexes protected with lactose exhibit similar particle size distribution to non-lyophilized lipid-pDNA complexes. The complexes protected with mannitol exhibit larger  
25 particle size distributions. As shown in FIG. 2, lipid-pDNA complexes lyophilized without a cryoprotectant aggregate while lipid-pDNA complexes protected with lactose or sucrose do not aggregate following rehydration and exhibit particles size distributions substantially the same as before lyophilization.

30 FIG. 3 shows the particle size distribution before and after lyophilization for various lipid-pDNA complexes protected with 10%

lactose. Lyophilization had little effect on particle size distribution regardless of the lipid composition or charge ratio.

FIG. 4 compares the zeta potential of lipid-pDNA complexes in the presence and absence of cryoprotectant. The presence of 10% lactose 5 had substantially no effect on the zeta potential, except at a charge ratio of 1:1.

FIG. 5 is a graphical representation of gel electrophoresis results comparing lipid-pDNA complexes before and after lyophilization. Migration of the bands was generally unaffected following lyophilization 10 and rehydration. This indicates the complexation between the lipid and the pDNA was not affected by the addition of 10% lactose.

FIGs. 6 and 7 show dose response curves of transfection efficiency comparing lyophilized and non-lyophilized lipid-pDNA complexes protected with 5% lactose, sucrose or glucose. At each 15 pDNA concentration and for each cryoprotectant, transfection efficiency was either unaffected or improved by lyophilization. FIGs. 8-10 show the effect of lyophilization on transfection efficiency for lipid-pDNA complexes: FIG. 8 shows transfer of CMV-CAT at pDNA:DOTMA/DOPE ratios of 1:10 and 1:6 in C<sub>2</sub> C<sub>12</sub> cells; FIG. 9 shows transfer of CMV- gal 20 at pDNA:DOTMA/DOPE ratios of 1:10 and 1:6 in C<sub>2</sub> C<sub>12</sub> cells; and FIG. 10 shows transfer of CMV- gal at pDNA:DOTMA/DOPE ratios of 1:10 and 1:6 in HepG2 cells. In each case transfection efficiency was improved by lyophilization.

FIGs. 11-13 show the transfection efficiency of stored 25 DDAB:DOPE-DNA lyophilized complexes prepared at various conditions. Storage of the lyophilized lipid-pDNA did not decrease the transfection efficiency and some cases activity increased. In contrast, FIG. 14 shows the effect of storage time on the activity of rehydrated lipid-pDNA complexes. Transfection efficiency fell over a two week period. 30 This indicates the DNA compositions are stable only when in a lyophilized condition.

Other useful DNA complexes may be prepared as follows:

1. A gramicidin S-pDNA complex is formed with DNA encoding the luciferase gene. At room temperature, 20  $\mu$ g of pDNA is diluted in 300  $\mu$ l of 30 mM Tris HCL pH 8.5 in a polystyrene tube. Gramicidin S is diluted in Tris HCL 30 mM ph 8.5 buffer to a concentration of 2 mg/ml from a stock solution of 20 mg/ml in dimethyl sulfoxide. The diluted gramicidin S (20  $\mu$ l/40  $\mu$ g) is added to the DNA and quickly mixed. Then 175  $\mu$ l liposomes (equivalent to 175 nmoles of lipids) are slowly added with gentle mixing to the DNA-gramicidin S mixture. Lactose is added to a final concentration of 225 mM and the material placed in a vial. The formulation is frozen in a dry-ice ethanol bath and then lyophilized to produce a dry cake. The dry cake may be stored at 4°C and rehydrated to original volume.
2. A dendrimer-pDNA complex is formed with DNA encoding the luciferase gene. 6  $\mu$ g of pDNA is diluted in 330  $\mu$ l of 10 mM Hepes pH 7.3 in a polystyrene tube. The polycation sixth generation starburst dendrimer (2 - 160  $\mu$ g) is diluted in Tris HCL 170 of HBS and added dropwise to the DNA and then gently mixed. Lactose is added to a final concentration of 225 mM and the material placed in a vial. The formulation is frozen in a dry-ice ethanol bath and then lyophilized to produce a dry cake. The dry cake may be stored at 4°C and rehydrated to original volume.

FIG. 15 shows expression in cells transfected using a  $\beta$ -gal-dendrimer complex cryoprotected with lactose or sucrose and lyophilized at various temperatures.

Using methods similar to those above, other useful lipid-polynucleotide complexes may be cryoprotected and lyophilized. FIG. 16 illustrates transfection using lyophilized complexes of  $\beta$ -gal associated with a 1:2 molar ratio of dimethyldioctadecylammonium bromide [DDAB]:dioleoyl phosphatidylethanolamine [DOPE] at varied

charge ratios and varied doses. The complexes were cryoprotected at a pDNA:lactose weight ratio of 1:15. FIG. 17 shows transfection using lyophilized complexes of  $\beta$ -gal associated with a 1:1 molar ratio of [DOTAP]:dioleyo phosphatidylethanolamine [DOPE] lyophilized with 5 various concentrations of sucrose and frozen at various temperatures.

In other embodiments, other cryoprotectants may be used at similar concentration to the above examples. By lyophilizing in the minimal concentration of cryoprotectant, the formulations can be lyophilized and then rehydrated in a lesser volume to concentrate 10 polynucleotide complex. The formulations may also include buffers that can be removed during lyophilization allows concentration of the preparation and subsequent rehydration to isotonicity. Suitable volatile buffers include triethanolamine-acetate, triethanolamine-carbonate, ammonium acetate, ammonium carbonate and other at concentrations 15 from about 0.01 M to about 2 M. For example, a polynucleotide complex in a 1.25% sucrose solution and a 100 mM ammonium triethanolamine carbonate may be lyophilized and then rehydrated to 1/8 the original volume, maintaining the isotonicity of the rehydrated solution and concentrating the polynucleotide complex 8-fold.

20 Cationic lipids are useful in forming complexes to be cryoprotected and lyophilized. Conventional cationic lipids suitable for the practice of the invention include phosphatidylethanolamine [PE], dioleyloxy phosphatidylethanolamine [DOPE], n-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride [DOTMA], 25 dioleoylphosphatidylcholine [DOPC], 2,3-dioleyloxy-N-[2-(sperminecarboxyamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate [DOSPA], [DOTAP], [DOGG], dimethyldioctadecylammonium bromide [DDAB], cetyltrimethylammonium bromide [CDAB], 30 cetyltrimethylammonium bromide [CTAB], monooleoyl-glycerol [MOG], 1,2 dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium

bromide [DMRIE], 1,2 dimyristoyl-sn-glycero-3-ethylphosphocholine [EDMPC], 1,2 dioleoyl-sn-glycero-3-ethylphosphocholine [EDOPC], 1 palmitoyl, 2 myristoyl-sn-glycero-3-ethylphosphocholine [EPMPC], cholesterol [Chol] and cationic bile salts. Other useful cationic lipids 5 may be prepared in the following manners.

**Spermine-5-carboxyglycine (N'-stearyl - N'-oleyl) amide tetr trifluoroacetic acid salt (JK-75) FIG. 19.**

A *p*-nitrophenyl oleinate ester was prepared by a standard method. This active ester coupled with octadecylamine to give N-octadecyl oleic 10 amide. Reduction of this amid by lithium aluminum hydride formed N-stearyl N-oleyl amine. A mixture of N-stearyl N-oleyl amine, N-butoxycarbonylglycine *p*-nitrophenyl ester, and triethylamine in dichloromethane was stirred at room temperature under argon for 24 h. The organic solution was extracted three times with 0.5 M sodium 15 carbonate, followed by water, and then dried over sodium sulfate. The solvent was removed under reduced pressure, and the residue was purified by a silica gel flash column to give N- t-butoxycarbonylglycine (N'-stearyl - N'-oleyl) amide. This compound was deprotected by trifluoroacetic acid to give glycine (N'-stearyl - N'-oleyl) amide, which 20 was then treated with tetra-t-butoxycarbonyl spermine-5-carboxylic acid (prepared by the cyanoethylation of ornitine, followed by a hydrogenation and protection with Boc-on), dicyclohexylcarbodiimide and N-hydroxysuccinimide in dichloromethane in dark at room temperature under argon for 48 h. The solvent was removed under 25 reduced pressure, and the residue was purified by a silica gel column. The desired compound was then deprotected in trifluoroacetic acid at room temperature for 10 min. The excess of acid was removed under vacuum to yield the spermine-5-carboxyglycine (N'-stearyl - N'-oleyl) amide tetra trifluoroacetic acid salt, as a light yellow wax. <sup>1</sup>H NMR (300 30 Mhz, CD<sub>3</sub>OD)  $\delta$  5.20 (m, 2 H), 4.01 (s, 2 H), 3.87 (t, 1 H), 3.19-2.90

## 12

(m, 16 H), 2.01-1.27 (m, 21 H), 1.15 (broad s, 56 H), 0.76 (t, 6 H). LSIMS (NBA): m/e 805.8 for  $M^{4+}$  ( $C_{49}H_{104}N_6O_2$ )-3H<sup>+</sup>.

**Spermine-5-carboxyglycine (N'-stearyl- N'-elaidyl) amide tetr trifluoroacetic acid salt (JK-76).** Fig 20.

5 Produced in a similar manner, by substituting for the appropriate starting material.

<sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD):  $\delta$  5.24 (m, 2 H), 4.01 (s, 2 H), 3.87 (t, 1 H), 3.14-2.90 (m, 16 H), 2.01-1.21 (m, 21 H), 1.15 (broad s, 56 H), 0.76 (t, 6 H). LSIMS (NBA): m/e 805.8 for  $M^{4+}$  ( $C_{49}H_{104}N_6O_2$ )-3H<sup>+</sup>

10 **Agmatinyl carboxycholesterol acetic acid salt (AG-Chol)** FIG. 21.

Agmatine sulfate (100 mg, 0.438 mmol) was treated by tetramethylammonium hydroxide (158 mg, 0.876 mmol) in methanol (15 mL) for 1 h. The solvent was removed under reduced pressure. A suspension solution of the residue and cholesteryl chloroformate (197 mg, 0.438 mmol) in DMF (15 mL) was stirred at room temperature for 3 days. Filtration of the reaction mixture gave the crude product as a light yellow solid, which was purified by a silica gel column using chloroform-methanol-acetic acid (10:2:1) as eluent to yield the agmatinyl carboxycholesterol acetic acid salt as a white solid. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD):  $\delta$  5.27 (broad s, 1 H), 4.65 (broad m, 1 H), 3.06 (t, 2 H), 2.99 (t, 2 H), 2.21 (broad d, 2 H), 1.95-0.65 (m, 31 H), 1.80 (s, 4 H), 0.91 (s, 3 H), 0.82 (d, 3 H), 0.76 (s, 3 H), 0.74 (s, 3 H), 0.59 (s, 3 H). LSIMS (NBA): m/e 543.4 for  $M^+$  ( $C_{33}H_{59}N_4O_2$ ).

15 **Spermine-5-carboxy- $\beta$ -alanine cholesteryl ester tetr trifluoroacetic acid salt (CAS)** FIG. 22.

20 A solution of cholesteryl  $\beta$ -alanine ester (0.2 mmol), prepared with standard procedure, in dichloromethane (dry, 2 mL) was added into a solution of tetra-t-butoxycarbonylspermine-5-carboxylic acid N-hydroxysuccinimide ester (0.155 mmol) and 4-methylmorpholine (0.4 mL) in dichloromethane (5 mL). The reaction mixture was stirred at room temperature under argon for 6 days. The solvent was removed

under reduced pressure, and the residue was purified by a silica gel column using ethanol-dichloromethane (1:20) as eluent to give the desired product as a light yellow oil. This compound was treated with trifluoroacetic acid (0.5 ml) at room temperature under argon for 10 5 min. The excess trifluoroacetic acid was removed under reduced pressure to give spermine-5-carboxy- $\beta$ -alanine cholesteryl ester tetr trifluoroacetic acid salt as a white solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  5.38 (m, 1 H), 4.60 (m, 1 H), 3.90 (t,  $J$  = 6.16, 1 H), 3.54 (m, 2 H), 3.04 (m, 10 H), 2.58 (t,  $J$  = 6.71, 2 H), 2.33 (d,  $J$  = 6.58, 10 2 H), 2.15 - 0.98 (m, 36 H), 1.04 (s, 3 H), 0.93 (d,  $J$  = 6.46, 3 H), 0.87 (d,  $J$  = 6.59, 6 H), 0.70 (s, 3 H). LSIMS (NBA): m/e 687.5 for 10  $\text{M}^{4+}$  ( $\text{C}_{41}\text{H}_{80}\text{N}_5\text{O}_3$ )-3H $^+$ .

**2,6-Diaminohexanoeyl  $\beta$ -alanine cholesteryl ester bistrifluoroacetic acid salt (CAL) FIG. 23.**

15 Produced in a manner similar to CAS, by substituting for the appropriate starting material.

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.10-7.62 (m, 7 H), 5.38 (broad s, 1 H), 4.60 (broad s, 1 H), 4.08 (broad s, 1 H), 3.40 (broad s, 4 H), 3.02 (broad s, 4 H), 2.50 (broad s, 2 H), 2.26 (broad s, 2 H), 2.04 - 0.98 (m, 20 28 H), 1.04 (s, 3 H), 0.93 (d,  $J$  = 6.46, 3 H), 0.88 (d,  $J$  = 6.59, 6 H), 0.74 (s, 3 H). LSIMS (NBA): m/e 586.5 for  $\text{M}^{2+}$  ( $\text{C}_{36}\text{H}_{65}\text{N}_3\text{O}_3$ )-H $^+$ .

**2,4-Diaminobutyroyl  $\beta$ -alanine cholesteryl ester bistrifluoroacetic acid salt (CAB) Fig. 24.**

Produced in a manner similar to CAS, by substituting for the 25 appropriate starting material.

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.34-8.06 (m, 7 H), 5.38 (broad s, 1 H), 4.60 (broad s, 1 H), 4.30-3.20 (broad m, 11 H), 2.50-0.98 (m, 36 H), 1.04 (s, 3 H), 0.93 (d,  $J$  = 6.46, 3 H), 0.88 (d,  $J$  = 6.59, 6 H), 0.74 (s, 3 H). LSIMS (NBA): m/e 558.5 for  $\text{M}^{2+}$  ( $\text{C}_{34}\text{H}_{61}\text{N}_3\text{O}_3$ )-H $^+$ .

30 **N, N-Bis (3-aminopropyl)-3-aminopropionyl  $\beta$ -alanine cholesteryl ester tristrifluoroacetic acid salt (CASD) FIG. 25.**

Cyanoethylation of the  $\beta$ -alanine with acrylnitrile in the presence of 1,4-diazabicyclo [2.2.2] octane at 90 °C for 15 h gave the N,N-bis(2-cyanoethyl)-3-aminopropionic acid. Hydrogenation of the N,N-bis(2-cyanoethyl)-3-aminopropionic acid in ethanol-water (1:1) using Raney 5 nickel as catalyst yielded the N,N-bis(3-aminoethyl)-3-aminopropionic acid. The amino groups of this compound was protected by 2-(t-butoxycarbonyloxyimino)-2-phenylacetonitrile in acetone-water (4:1) to give N,N-bis (t-butoxycarbonyl-3-aminoethyl)-3-aminopropionic acid. This compound was activated by chloroacetonitrile and triethylamine to 10 form cyanomethyl N,N-bis (t-butoxycarbonyl-3-aminoethyl)-3-aminopropionate. A solution of the cyanomethyl ester and cholesteryl  $\beta$ -alanine ester in chloromethane was stirred in dark at room temperature under argon for 10 days. The solvent was removed under reduced pressure, and the residue was purified by a silica gel column using 15 methanol-chloroform (1:10) as eluant to yield the N,N-bis (t-butoxycarbonyl-3-aminoethyl)-3-aminopropionyl  $\beta$ -alanine cholesteryl ester. Treatment of this compound with trifluoroacetic acid formed N, N-bis (3-aminopropyl)-3-aminopropionyl  $\beta$ -alanine cholesteryl ester 20 trifluoroacetic acid salt.  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}-\text{CDCl}_3$  1:1):  $\delta$  8.13 (broad s, 3 H), 5.78 (broad s, 3 H), 5.38 (broad s, 1 H), 5.18 (s, 1H), 4.74 (s, 1 H), 4.60 (broad s, 1 H), 3.54-3.04 (m, 10 H), 2.80 (t,  $J=6.60$ , 2 H), 2.73 (t,  $J=6.54$ , 2 H), 2.53(t,  $J=6.42$ , 2 H), 2.32(d,  $J = 6.58$ , 2 H), 2.15 - 0.98 (m, 30 H), 1.04 (s, 3 H), 0.91 (d,  $J = 6.42$ , 3 H), 0.86 (d,  $J = 6.58$ , 6 H), 0.70 (s, 3 H). LSIMS (NBA): m/e 25 643.5 for  $\text{M}^{3+} (\text{C}_{39}\text{H}_{73}\text{N}_4\text{O}_3)-2\text{H}^+$ .

**[N, N-Bis(2-hydroxyethyl)-2-aminoethyl]aminocarboxy cholesteryl ester (JK-154) FIG. 26.**

A solution of cholesteryl chloroformate (0.676 g, 1.51 mmol) and ethylenediamine (4 mL) in chloroform (10 mL) was stirred in dark at 30 room temperature under argon for 16 h. The solvent and excess of

ethylenediamine were removed under reduced pressure, and the residue was purified by a silica gel column using  $\text{CH}_3\text{OH}-\text{CHCl}_3$  ( $\text{NH}_3$ ) (v/v, 0-20 %) as eluent to give ethylenediamine cholesterylcarboxymonoamide as a white solid. A mixture of this compound (80 mg, 0.17 mmol), 2-

5 hydroxyethylbromide (2 mL) and triethylamine (2 mL) was stirred in dark at room temperature under argon for 14 days. The excess of triethylamine and 2-hydroxyethylbromide were removed under reduced pressure, and the residue was purified by a silica gel column using  $\text{CH}_3\text{OH}-\text{CHCl}_3$  (v/v, 1:3) as eluent to give the 2-[N, N-Bis(2-hydroxyethyl) 10 aminoethyl] amino carboxy cholesteryl ester as a white solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  LSIMS (NBA): m/e 643.5 for  $\text{M}^{3+}$  ( $\text{C}_{39}\text{H}_{73}\text{N}_4\text{O}_3$ )-2H<sup>+</sup>.

#### **Carnitine ester lipids**

Carnitine lipids are synthesized by acylating the hydroxy group of L-

15 carnitine by standard methods to create the monoacyl carnitine. The carboxy group of the carnitine is modified with a second acyl chain to make a phospholipid analog with a single quarternary ammonium cationic group. The other carnitine stereoisomers D- and D,L- are suitable, but the L-form is preferred. The acyl chains are between 2 and 20 30 carbons and may be saturated or unsaturated, with from 1 to 6 unsaturated groups in either the cis or trans configuration. The acyl chains may also contain iso forms. A preferred form comprises the oleoyl group, a chain 18 carbons long with a cis unsaturated bond at C<sub>9</sub>. This generic carnitine ester is shown in FIG. 27. Presently preferred 25 carnitine esters follow.

#### **Stearyl carnitine ester**

A solution of DL-carnitine hydrochloride (1.0 g, 5.05 mmol) and sodium

hydroxide (0.303 g, 7.58 mmol) in ethanol (15 mL) was stirred at room temperature for 2 h. The formed white precipitate (NaCl) was removed

30 by filtration, and the solvent was evaporated under reduced pressure to give a white solid, carnitine inner salt. A suspension of the carnitine

inner salt and 1-iodooctadecane (2.31 g, 6.06 mmol) in DMF-dioxane (3 : 5, 40 mL) was heated with an oil-bath at 120 °C under Ar<sub>2</sub> for 4 h. The solvent was removed by rotavapor and vacuum, and the residue was chromatographed with silica gel column using CH<sub>3</sub>OH-CH<sub>3</sub>Cl as 5 eluant to give 2.22 g (81 %) of stearyl carnitine ester as a white solid:  
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 4.79 (m, 1 H), 4.43 (d, J = 5.3, 1 H), 4.09 (t, J = 6.9, 2 H), 4.03 (d, J = 13.0, 1 H), 3.67 (dd, J = 10.3, 13.3, 1 H), 3.51 (s, 9 H), 2.79 (dd, J = 5.7, 17.0, 1 H), 2.66 (dd, J = 7.0, 17.1, 1 H), 1.80-1.60 (m, 4 H), 1.26 (broad s, 28 H), 0.88 (t, J = 6.6, 3 H).  
10 LSIMS (NBA): m/e 414.4 for C<sub>25</sub>H<sub>52</sub>NO<sub>3</sub> (cation).

#### Palmityl carnitine ester

With the procedure used for the preparation of stearyl carnitine ester, 0.77 g (4.77 mmol) of carnitine inner salt and 2.52 g (7.15 mmol) of 1-iodohexadecane to give 1.59 g (65 %) of palmityl carnitine ester as a 15 white solid: <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 4.78 (m, 1 H), 4.44 (d, J = 5.4, 1 H), 4.09 (t, J = 6.9, 2 H), 3.65 (dd, J = 10.2, 13.3, 1 H), 3.58 (d, J = 5.1, 1 H), 3.51 (broad s, 9 H), 2.80 (dd, J = 5.7, 17.2, 1 H), 2.66 (dd, J = 7.1, 17.1, 1 H), 1.65 (broad m, 4 H), 1.26 (broad s, 24 H), 0.88 (t, J = 0.66, 3 H). LSIMS (NBA): m/e 386.2 for C<sub>23</sub>H<sub>48</sub>NO<sub>3</sub> (cation).

#### 20 Myristyl carnitine ester

With the procedure used for the preparation of stearyl carnitine ester, 0.77 g (4.77 mmol) of carnitine inner salt and 2.31 g (7.15 mmol) of 1-iodotetradecane gave 1.70 (74 %) of myristyl carnitine ester as a white solid: <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 4.79 (m, 1 H), 4.43 (d, J = 5.3, 1 H), 4.09 (t, J = 6.9, 2 H), 4.03 (d, J = 13.0, 1 H), 3.67 (dd, J = 10.3, 13.3, 1 H), 3.51 (s, 9 H), 2.79 (dd, J = 5.7, 17.0, 1 H), 2.66 (dd, J = 7.0, 17.1, 1 H), 1.80-1.60 (m, 4 H), 1.26 (broad s, 20 H), 0.88 (t, J = 6.6, 3 H). LSIMS (NBA): m/e 358.1 for C<sub>21</sub>H<sub>44</sub>NO<sub>3</sub> (cation).

#### Stearyl stearoyl carnitine ester chloride salt (SSCE) FIG. 28.

30 A solution of DL-carnitine hydrochloride (1.0 g, 5.05 mmol) and sodium hydroxide (0.303 g, 7.58 mmol) in ethanol (15 mL) was stirred

at room temperature for 2 h. The formed white precipitate (NaCl) was removed by filtration, and the solvent was evaporated under reduced pressure to give a white solid, carnitine inner salt. A suspension of the carnitine inner salt and 1-iodooctadecane (2.31 g, 6.06 mmol) in DMF-

5 dioxane (3 : 5, 40 mL) was heated with an oil-bath at 120 °C under argon for 4 h. The solvent was removed under reduced pressure, and the residue was purified by a silica gel column using  $\text{CH}_3\text{OH}-\text{CH}_3\text{Cl}$  (v/v, 0-10%) as eluent to give the stearyl carnitine ester as a white solid. A solution of a fresh prepared stearic anhydride (1.94 g, 3.52 mmol),  
10 stearyl carnitine ester (0.953 g, 1.76 mmol) and 4-dimethylaminopyridine (0.429 g, 3.52 mmol) in  $\text{CH}_3\text{Cl}$  (dry, 15 mL) was stirred at room temperature under argon for four days. The solvent was removed under reduced pressure, and the residue was washed twice by cold diethyl ether. The solid was chromatographed on a silica gel  
15 column using  $\text{MeOH}-\text{CHCl}_3$  (v/v, 1:5) as eluent to give the stearyl stearoyl carnitine ester iodide. The iodide was exchanged by chloride with an anion exchange column to give the stearyl stearoyl carnitine ester chloride as a white solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  5.67 (q, 1 H), 4.32 (d, 1 H), 4.07 (m, 3 H), 3.51 (s, 9 H), 2.82 (t, 2 H), 2.33 (t, 2 H), 1.59 (broad m, 4 H), 1.25 (broad s, 58 H), 0.88 (t, 6 H). LSIMS (NBA): m/e 680.6 for  $\text{M}^+$  ( $\text{C}_{43}\text{H}_{86}\text{NO}_4$ ). Anal. Calcd for  $\text{C}_{43}\text{H}_{86}\text{ClNO}_4 \cdot \text{H}_2\text{O}$ : C, 70.30; H, 12.07; N, 1.91. Found: C, 70.08; H, 12.24; N, 1.75.

L-Stearyl Stearoyl Carnitine Ester (L-SSCE) was prepared with the same

25 procedure using L-carnitine as starting material. Analytical data are same as DL-SSCE.

**Stearyl oleoyl carnitine ester chloride (SOCE) FIG. 29.**

Prepared in a manner similar to SSCE, by substituting the appropriate starting material.

30  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  5.67 (q, 1 H), 5.35 (m, 2 H), 4.32 (d, 1 H), 4.08 (m, 3 H), 3.48 (s, 9 H), 2.83 (dd, 2 H), 2.34 (dd, 2 H), 2.02

(broad m, 4 H), 1.26 (broad m, 54 H), 0.88 (t, 6 H). LSIMS (NBA): m/e 678.7 for  $M^+$  ( $C_{43}H_{84}NO_4$ ). Anal. Calcd for  $C_{43}H_{84}ClNO_4 \cdot H_2O$ : C, 70.50; H, 11.83; N, 1.91. Found: C, 70.77; H, 12.83; N, 1.93.

**Palmityl palmitoyl carnitine ester chloride (PPCE) FIG. 30.**

5 Prepared in a manner similar to SSCE, by substituting the appropriate starting material.

10  $^1H$  NMR (300 MHz,  $CDCl_3$ ):  $\delta$  5.67 (q, 1 H), 4.33 (d, 1 H), 4.07 (m, 3 H), 3.51 (s, 9 H), 2.82 (t, 2 H), 2.33 (t, 2 H), 1.59 (broad m, 4 H), 1.25 (broad s, 58 H), 0.99 (t, 6 H). LSIMS (NBA): m/e 680.6 for  $M^+$  ( $C_{43}H_{78}NO_4$ ). Anal. Calcd for  $C_{39}H_{78}ClNO_4 \cdot H_2O$ : C, 69.04; H, 11.88; N, 2.06. Found: C, 69.31; H, 11.97; N, 2.37.

**Myristyl myristoyl carnitine ester chloride (MMCE) FIG. 31.**

Prepared in a manner similar to SSCE, by substituting the appropriate starting material.

15  $^1H$  NMR (300 MHz,  $CDCl_3$ ):  $\delta$  5.67 (q, 1 H), 4.32 (d, 1 H), 4.07 (m, 3 H), 3.50 (s, 9 H), 2.82 (t, 2 H), 2.33 (t, 2 H), 1.61 (broad m, 4 H), 1.26 (broad s, 42 H), 0.88 (t, 6 H). LSIMS (NBA): m/e 568.6.7 for  $M^+$  ( $C_{35}H_{70}NO_4$ ). Anal. Calcd for  $C_{35}H_{70}ClNO_4 \cdot 1/2H_2O$ : C, 68.53; H, 11.67; N, 2.28. Found: C, 68.08; H, 11.80; N, 2.21.

20 **L-Myristyl myristoyl carnitine ester chloride (L-MMCE)** was prepared with the same procedure using L-carnitine as starting material. Analytical data are same as DL-MMCE. m.p. 157 °C (decomposed).

These results demonstrate a number of the benefits exhibited by lyophilized polynucleotide complexes. The freeze-drying process does not substantially effect the physicochemical properties of the polynucleotide complexes yet confer stability over protracted periods of time. The formulations allow preparation of high concentrations of complex. For cationic lipids at least, lyophilization with certain cryoprotectants followed by rehydration results in improved transfection efficiencies compared to non-lyophilized controls. It is believed that the

process stabilizes the polynucleotide-cation interaction, generating complexes of defined particle size following rehydration.

#### Dry Powder Formulations

5        Lyophilized polynucleotide complexes may be sieved or milled to produce dry powder formulations (DPF). The powder may be used to generate a powder aerosol for delivering the polynucleotide to the lung. A current limitation of aerosol delivery is that high concentrations of DNA must be used in order to achieve sufficient gene transfer. At these 10 concentrations, the polynucleotide complexes aggregate. The DPF permits use of high concentrations of polynucleotide. The powder is diluted when dispersed into the lung so that risk of aggregation is minimized. Once in contact with the lung tissue, the powder will rehydrate and regain its activity.

15       In a first example, plasmid CMV-CAT DNA was complexed as described above with a DOTMA:DOPE lipid formulation at a ratio of 1:10 (w/w). As a control, naked pCMV-CAT may be cryoprotected. The cryoprotectant mannitol was added at pDNA:mannitol ratios of 1:100 and 1:500 (w/w). The formulations were lyophilized at a product eutect 20 temperature of 30°C to form a dry cake and retained under vacuum. Control instillation formulations were rehydrated to provide physicochemical and transfective comparison to the DPFs. The lyophilized product was sieved using a brass U.S.A. Standard 38 µm Sieve Apparatus in a dry glove box. The complex powder was sieved 25 into crystalline lactose (Pharmatose ) to produce a 1:6 (w/w) powder:Pharmatose concentration. Pharmatose acts as a carrier for the DPF formulations. In other embodiments, a carrier may not be desirable. Resulting exemplary pCMV-CAT concentrations for the DPFs are as follows:

30       1:0:100 (w/w/w) pDNA:lipid:mannitol      -      1.41 µg/mg  
            1:10:100 (w/w/w) pDNA:lipid:mannitol      -      1.29 µg/mg

## 20

|                                      |   |                 |
|--------------------------------------|---|-----------------|
| 1:0:500 (w/w/w) pDNA:lipid:mannitol  | - | 0.29 $\mu$ g/mg |
| 1:10:500 (w/w/w) pDNA:lipid:mannitol | - | 0.28 $\mu$ g/mg |

The DPFs were tested for *in vivo* activity by treating mice with the formulations and a Pharmatose control, harvesting the lung and trachea 5 and assessing CAT expression. 10 mg of DPF were delivered via direct intratracheal injections using a Penn-Century Delivery device, resulting in approximately 50% delivery. FIG. 18 shows that the CMV-CAT DPF delivered at various doses resulted in CAT expression in the lung cells.

In another example, similar DPFs were produced using a jet milled 10 using a high speed shear mixer. A 1:10:100 (w/w/w) pDNA/lipid/mannitol complex was jet milled at a grinding pressure of 130 psi and a feed rate of 40 mg/ml. The resulting powder had a nearly monodisperse particle size distribution of 80% at 3.2 - 3.8  $\mu$ m as determined by laser light scattering. Electron microscopy revealed that 15 many particles were <1  $\mu$ m. Jet milling at a grinding pressure of 80 psi and a feed rate of 700 mg/ml resulted in a nearly monodisperse particle size distribution of 80% at 3.7 - 4.8  $\mu$ m. In comparison, a sieved DPF showed a slight increase in the percentage of particles <10  $\mu$ m. Prior to jet milling, the DPF was polydisperse with a particle size distribution 20 of 80% at 5 - 27  $\mu$ m.

DPFs may be used to deliver genes useful in the treatment of a lung disease. For example, complexes formed with DNA encoding for 25 cystic fibrosis transmembrane conductance regulator (CFTR) may be used to treat cystic fibrosis. In similar manner, other lung diseases such as alpha-1-antitrypsin deficiency, asthma, pulmonary embolism, adult respiratory distress syndrome, pulmonary hypertension, chronic obstructive pulmonary disease, lung cancer, pulmonary fibrosis, pulmonary microbial infections, pulmonary pseudomonas infections, pulmonary inflammatory disorders, chronic bronchitis, pulmonary viral 30 infections, respiratory syncitial virus, lung tissue rejection, emphysema and pulmonary allergic disorders could be treated. In preferred

embodiments, the average particle size of the DPF is controlled to skew the deposition of the particles in desired region of the respiratory system. Although the deposition of particles is affected by a number of factors, including environmental conditions, particle characteristics, 5 respiratory tract characteristics and breathing pattern characteristics, predictive models are possible. FIG. 32 shows the deposition fraction at various compartments of the respiratory tract for inhaled aerosols as a function of particle size. DPFs should generally have an average particle size of less than about 100  $\mu\text{m}$ , preferably less than about 10 10  $\mu\text{m}$ , and particularly preferably less than about 1  $\mu\text{m}$  for treatment of the lung.

In other embodiments, DPFs are useful for the treatment of skin diseases. DPFs could be also be formulated as a pill for ingestion or as a suppository allowing for treatment of a wide range of internal or 15 systemic conditions.

## What is claimed is:

1. A composition for delivering a polynucleotide to a cell, comprising a lyophilized formulation of a polynucleotide complex and a 5 cryoprotectant.
2. The composition of claim 1, wherein the cryoprotectant comprises a carbohydrate.
- 10 3. The composition of claim 2, wherein the carbohydrate is selected from the group consisting of lactose, sucrose, glucose, maltodextrins, mannitol, sorbitol, trehalose and butanes.
- 15 4. The composition of claim 3, comprising a polynucleotide:carbohydrate weight ratio from about 1:10 to 1:500.
- 20 5. The composition of claim 4, comprising a weight ratio of about 1:100.
- 25 6. The composition of claim 1, wherein the polynucleotides complex is selected from the group consisting of polynucleotides, multiple polynucleotides, plasmid DNA, polynucleotides associated with a lipid, polynucleotides associated with a cationic lipid, polynucleotides associated with a carbohydrate, polynucleotides associated with a liposome or lipidic particle, polynucleotides associated with a cationic peptide polynucleotides associated with a polycation, polynucleotides associated with a polylysine, polynucleotides associated with a dendrimer, and polynucleotides associated with a branched polycation.
- 30 7. The composition of claim 6, wherein the polynucleotide associated with a lipid comprises a lipid selected from the group

consisting phosphatidylethanolamine [PE], phosphatidyl choline [PC], dioleyloxy phosphatidylethanolamine [DOPE], n-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride [DOTMA], dioleoylphosphatidylcholine [DOPC], 2,3-dioleyloxy-N-[2-(sperminecarboxyamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate [DOSPA], [DOTAPI], [DOGG], spermine-5-carboxyglycine(N'-stearyl-N'-stearyl)amide tetra-trifluoroacetic acid salt [DOGS], 1,2 dimyristyloxypropyl-3-dimehtyl-hydroxyethyl ammonium bromide [DMRIE], 1,2 dimyristoyl-sn-glycero-3-ethylphosphocholine [EDMPC], 1,2 dioleoyl-sn-glycero-3-ethylphosphocholine [EDOPC], 1 palmitoyl, 2 myristoyl-sn-glycero-3-ethylphosphocholine [EPMPC] dimethyl dioctadecyl ammonium bromide [DDAB], cetyl dimethyl ethyl ammonium bromide [CDAB], cetyltrimethyl ethyl ammonium bromide [CTAB], monooleoyl-glycerol [MOG], cholesterol [Chol], cationic bile salts, spermine-5-carboxyglycine (N'-stearyl - N'-oleyl) amide tetratrifluoroacetic acid salt [JK-75], spermine-5-carboxyglycine (N'-stearyl- N'-elaidyl) amide tetratrifluoroacetic acid salt [JK-76], agmatinyl carboxycholesterolacetic acid salt [AG-Chol], spermine-5-carboxy- $\beta$ -alanine cholesteryl ester tetratrifluoroacetic acid salt [CAS], 2,6-diaminohexanoeyl  $\beta$ -alanine cholesteryl ester bistrifluoroacetic acid salt [CAL], 2,4-diaminobutyroyl  $\beta$ -alanine cholesteryl ester bistrifluoroacetic acid salt [CAB], N, N-bis (3-aminopropyl)-3-aminopropionyl  $\beta$ -alanine cholesteryl ester tristrifluoroacetic acid salt [CASD], [N, N-bis(2-hydroxyethyl)-2-aminoethyl]aminocarboxy cholesteryl ester [JK-154], carnitine ester lipids, steryl carnitine ester, myristyl carnitine ester, steryl stearoyl carnitine ester chloride salt [SSCE], L-stearyl stearoyl carnitine ester [L-SSCE], steryl oleoyl carnitine ester chloride [SOCE], palmitoyl palmitoyl carnitine ester chloride [PPCE], myristyl myristoyl carnitine ester chloride [MMCE], L-myristyl myristoyl carnitine ester chloride [L-MMCE].

8. The composition of claim 6, wherein the complex further comprises a transfecting effector selected from the group consisting of a lipid, a cationic lipid, a carbohydrate, a liposome or lipidic particle, a cationic peptide, a polycation, a polylysine, a dendrimer and a branched 5 polycation.

9. The composition of claim 7, wherein the polynucleotide complex comprises a polynucleotide associated with [DOTAP]:dioleoyl phosphatidylethanolamine [DOPE].

10

10. The composition of claim 7, wherein the polynucleotide complex comprises a polynucleotide associated with dimethyldioctadecylammonium bromide [DDAB]:dioleoyl phosphatidylethanolamine [DOPE].

15

11. The composition of claim 6, wherein the polynucleotide complex comprises a polynucleotide associated with an amphipathic peptide.

20

12. The composition of claim 11, wherein the amphipathic peptide comprises gramicidin S.

13. The composition of claim 6, wherein the polynucleotide complex comprises a polynucleotide associated with a dendrimer.

25

14. A method for treating a polynucleotide complex comprising the steps of adding a cryoprotectant to the polynucleotide complex and then lyophilizing the polynucleotide complex.

30

15. The method of claim 14, wherein the step of adding a cryoprotectant comprises adding a carbohydrate selected from the group

consisting of lactose, sucrose, glucose, maltodextrins, mannitol, sorbitol, trehalose and butanes.

16. The method of claim 14, wherein the step of lyophilizing  
5 the polynucleotide complex comprises freezing the complex at a  
temperature from about -20°C to about -80°C.

17. The method of claim 14, wherein the step of lyophilizing  
the polynucleotide complex comprises freezing the complex at the  
10 eutectic temperature of the complex.

18. The method of claim 14, wherein the step of adding a  
cryoprotectant comprises adding a carbohydrate to produce a  
polynucleotide:cryoprotectant weight ratio from about 1:10 to about  
15 1:500.

19. The method of claim 18, wherein the step of adding a  
cryoprotectant comprises adding lactose to produce a  
polynucleotide:cryoprotectant weight ratio of about 1:100.  
20

20. The method of claim 14, further comprising the step of  
rehydrating the complex after lyophilization.

21. A method for delivering a polynucleotide to a cell  
25 comprising the steps of adding a cryoprotectant to a polynucleotide  
complex, lyophilizing the complex and contacting the cell with the  
complex.

22. The method of claim 21, further comprising the step of  
30 rehydrating the complex before contacting the cell.

23. The method of claim 22, wherein the step of rehydrating the complex comprises rehydrating the complex to a lower volume to concentrate the complex.

5 24. The method of claim 23, further comprising the step of adding a volatile buffer to the complex before lyophilizing.

25. A method for gene therapy comprising contacting a eukaryotic cell with the composition of claim 1.

10

26. The method of claim 25 comprising contacting the cell under *in vivo* conditions.

15 27. The method of claim 25 comprising contacting the cell under *in vitro* conditions.

28. The method of claim 26 comprising contacting a mammalian cell.

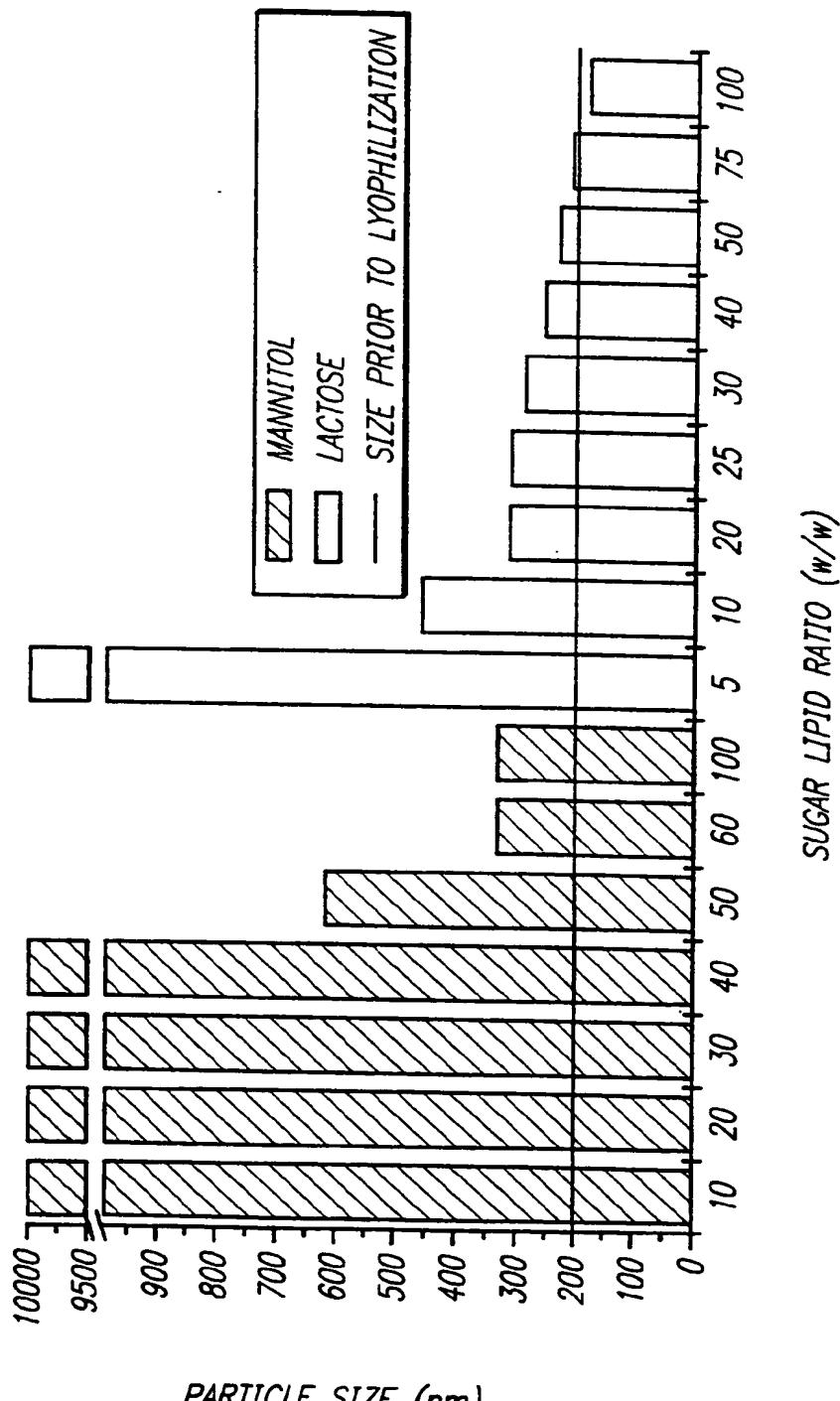
20 29. The method of claim 26 comprising contacting a human cell.

30. The method of claim 27 comprising contacting a mammalian cell.

25

31. The method of claim 27 comprising contacting a human cell.

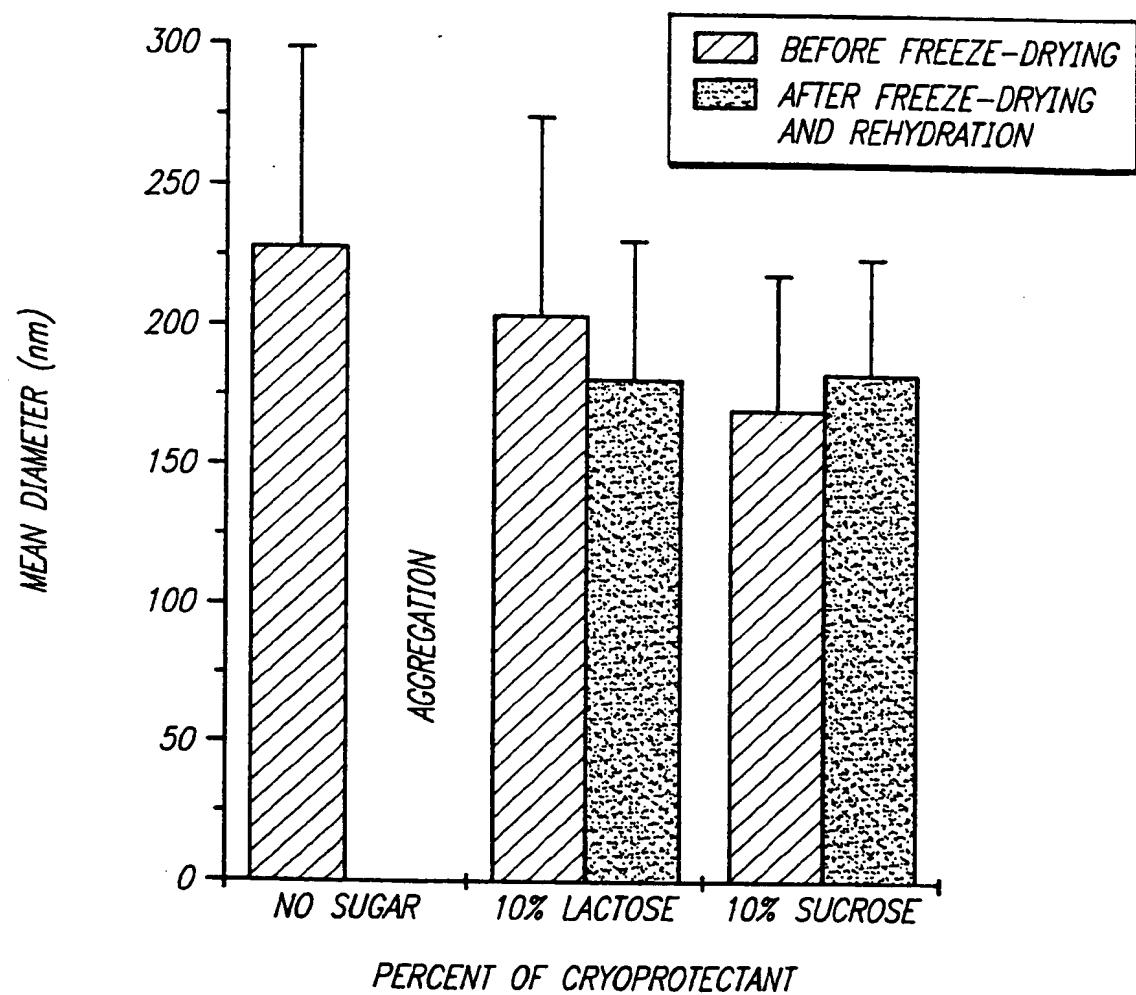
FIG. 1



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FIG. 2



- \* DNA/DOTMA:DOPE RATIO = 1:10 w/w
- \* LIPOSOME:SUGAR RATIO = 1:100 w/w
- \* DNA CONCENTRATION = 100  $\mu$ g/mL
- \* SIMILAR RESULTS WERE OBTAINED WITH 5% CRYOPROTECTANT.

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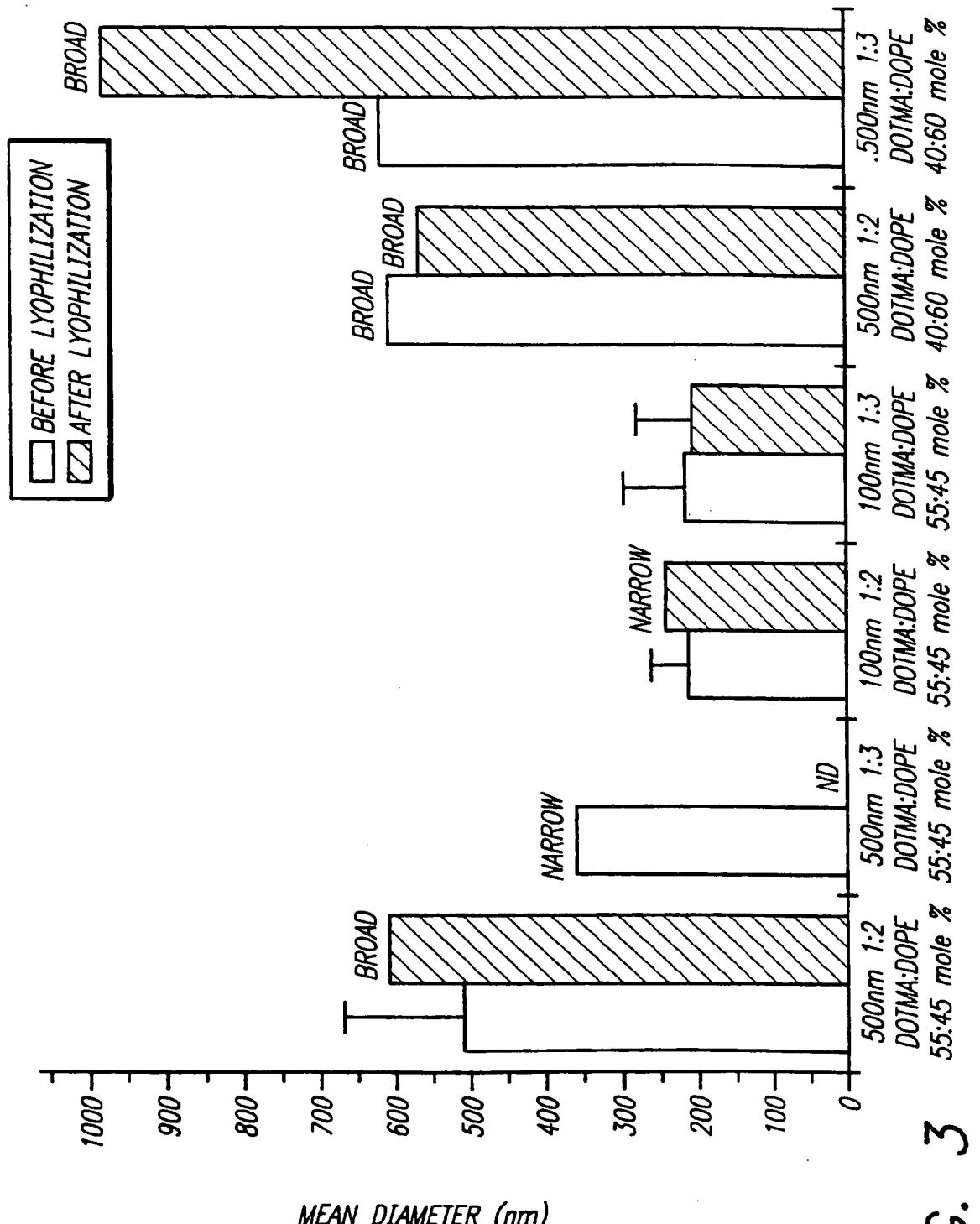


FIG. 3

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FORMULATION: -/+ CHARGE RATIO, SIZE OF LIPOSOMES

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FIG. 4

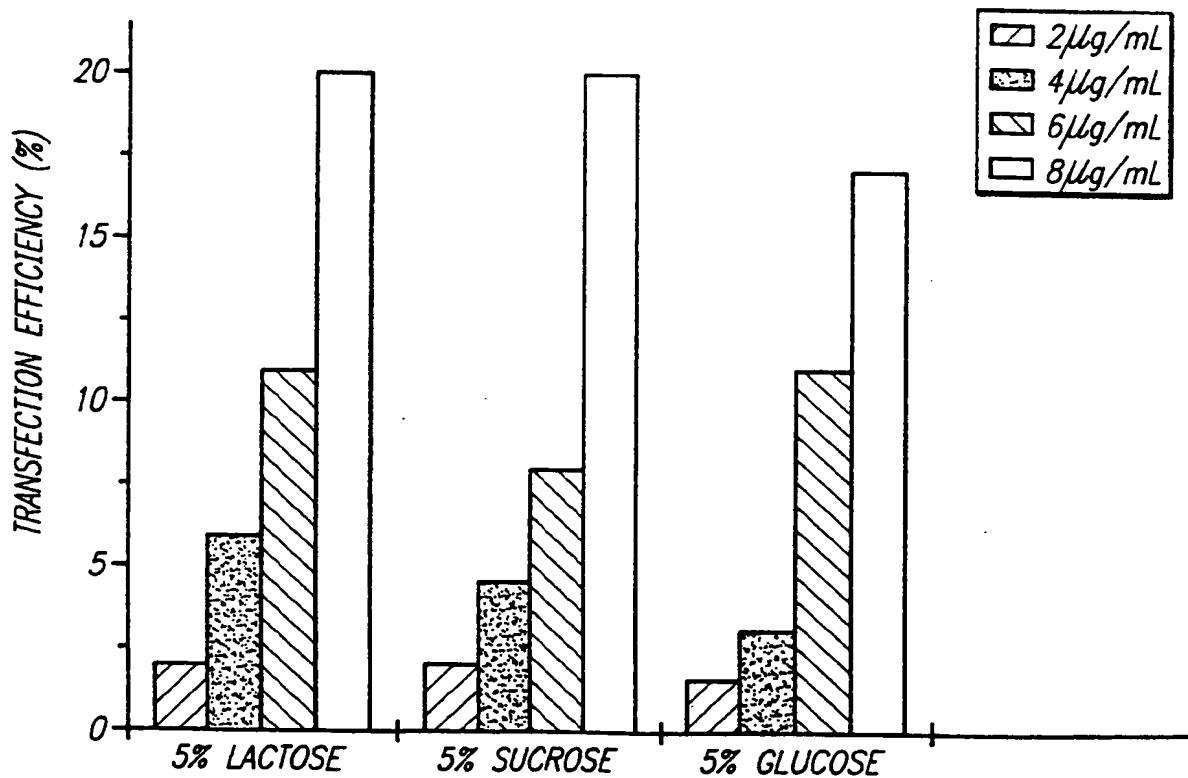
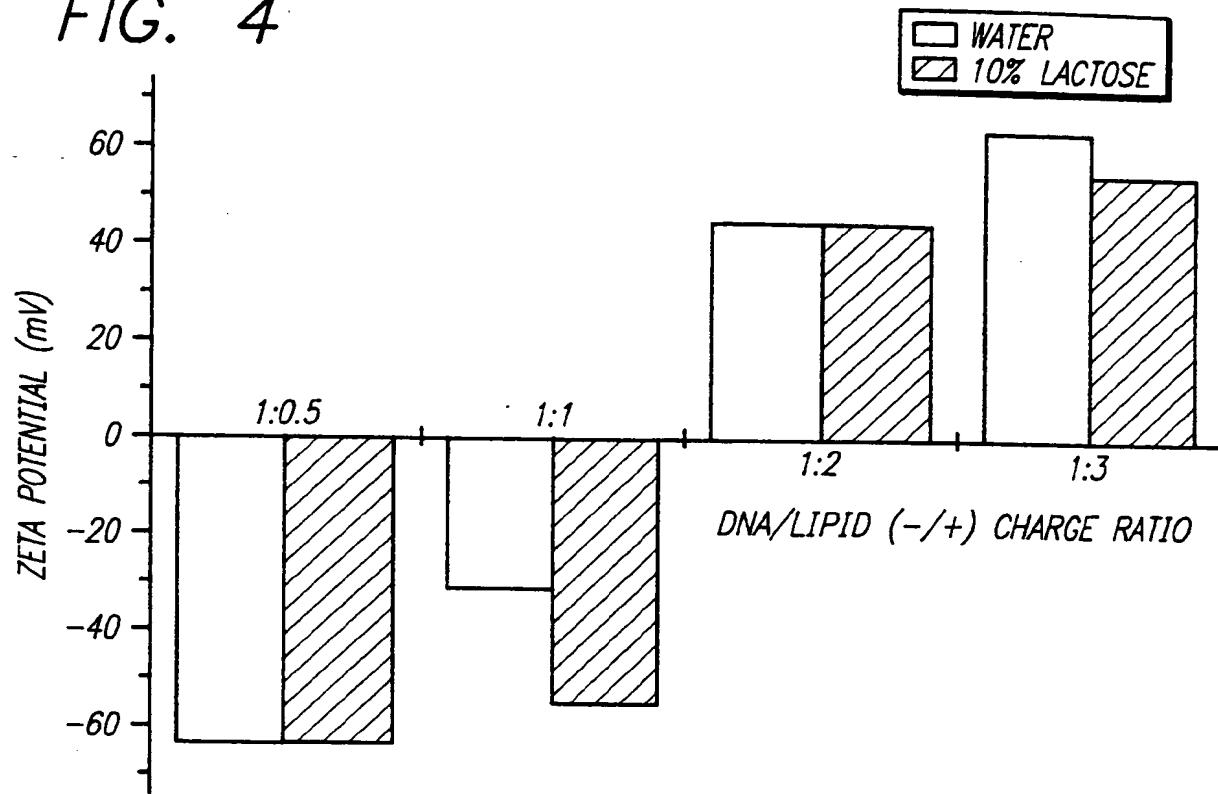


FIG. 6

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1% AGAROSE GEL ELECTROPHORESIS OF DNA/LIPID COMPLEX  
IN THE PRESENCE OF 10% LACTOSE:

BEFORE LYOPHILIZATION

LANE 1: 1:2 -/+ , DOTMA:DOPE, 55:45 mol%, 100nm  
LANE 2: 1:2 -/+ , DOTMA:DOPE, 40:60 mol%, 100nm  
LANE 3: 1:3 -/+ , DOTMA:DOPE, 40:60 mol%, 100nm  
LANE 4: 1:0.5 -/+ , DOTMA:DOPE, 55:45 mol%, 100nm  
LANE 5: FREE DNA  
LANE 6: MOLECULAR STANDARD

AFTER LYOPHILIZATION AND REHYDRATION

LANE 7: 1:2 -/+ , DOTMA:DOPE, 55:45 mol%, 100nm  
LANE 8: 1:3 -/+ , DOTMA:DOPE, 40:60 mol%, 100nm  
LANE 9: 1:3 -/+ , DOTMA:DOPE, 40:60 mol%, 100nm  
LANE 10: 1:0.5 -/+ , DOTMA:DOPE, 55:45 mol%, 100nm

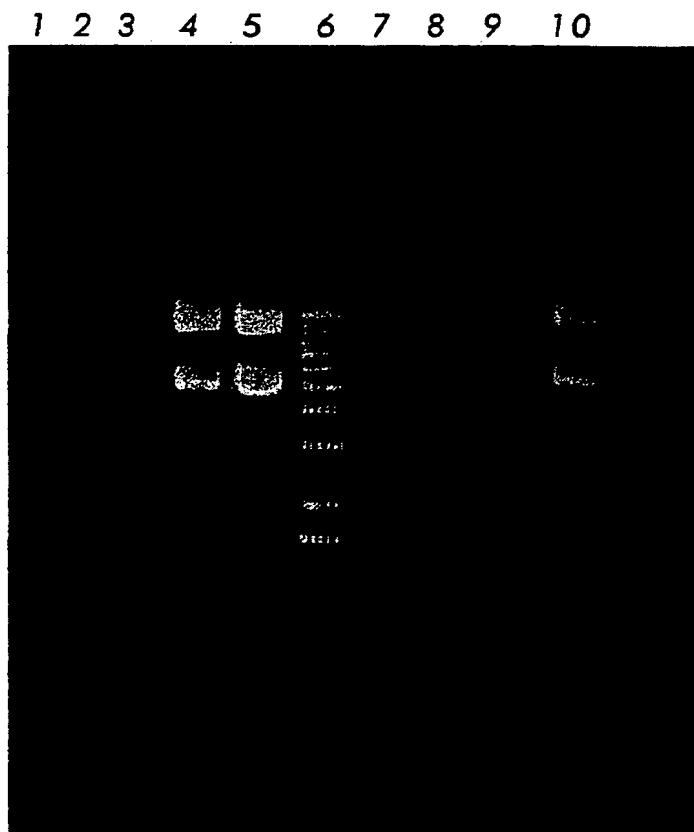
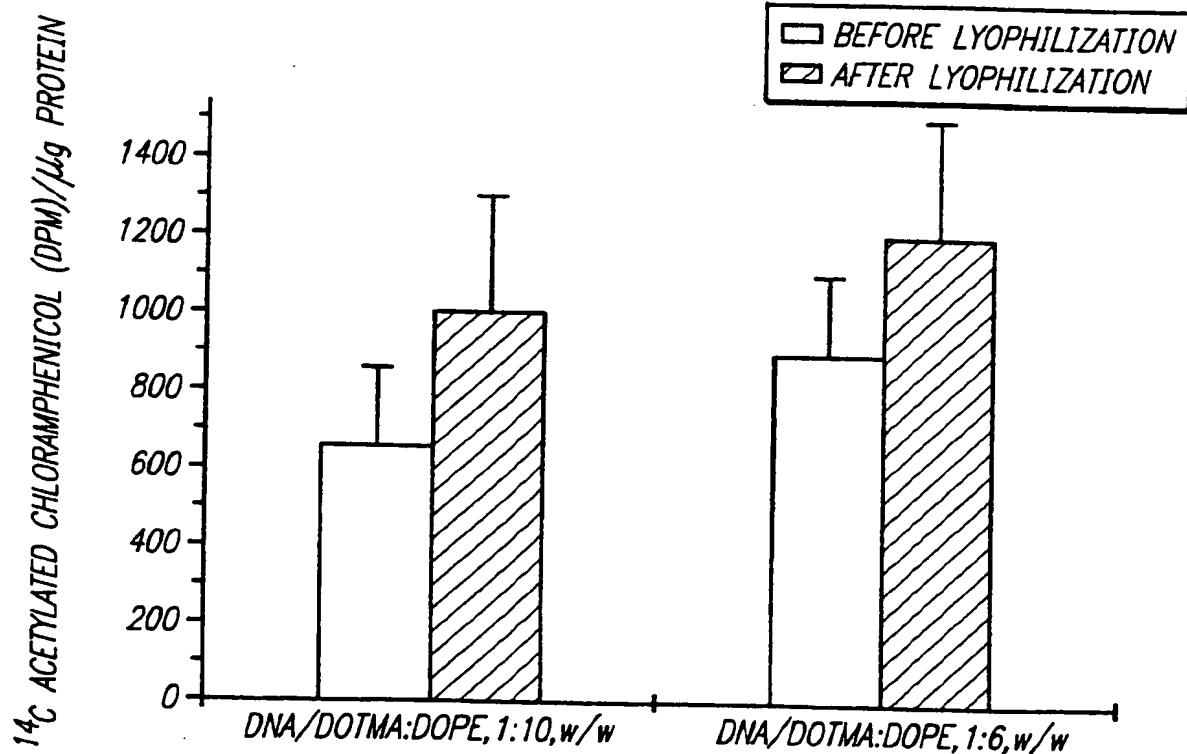
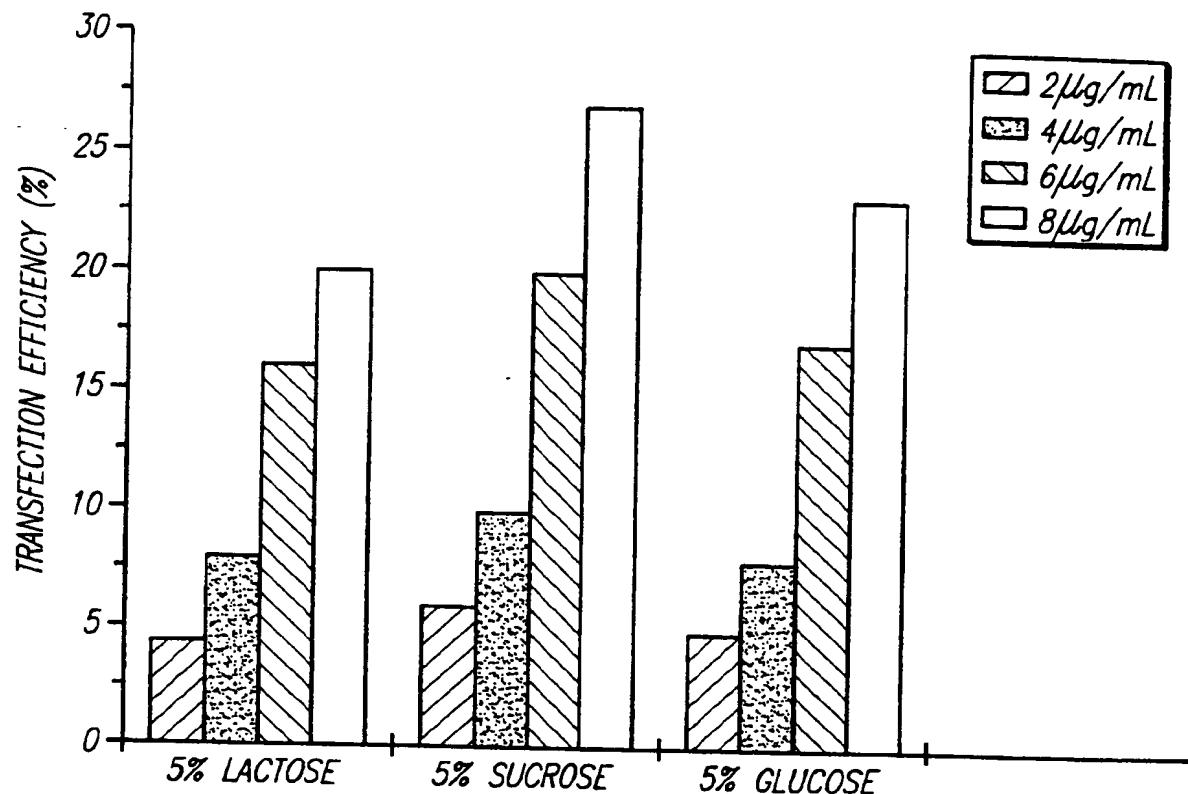


FIG. 5

SUBSTITUTE SHEET (RULE 26)

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FIG. 7

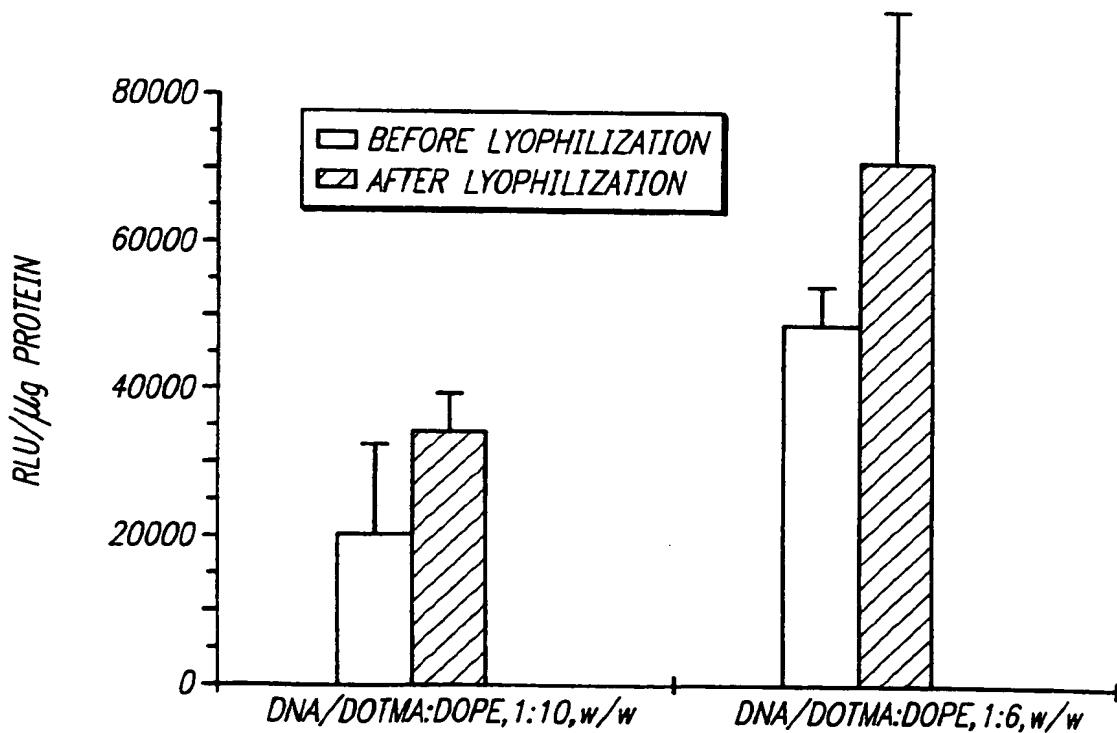
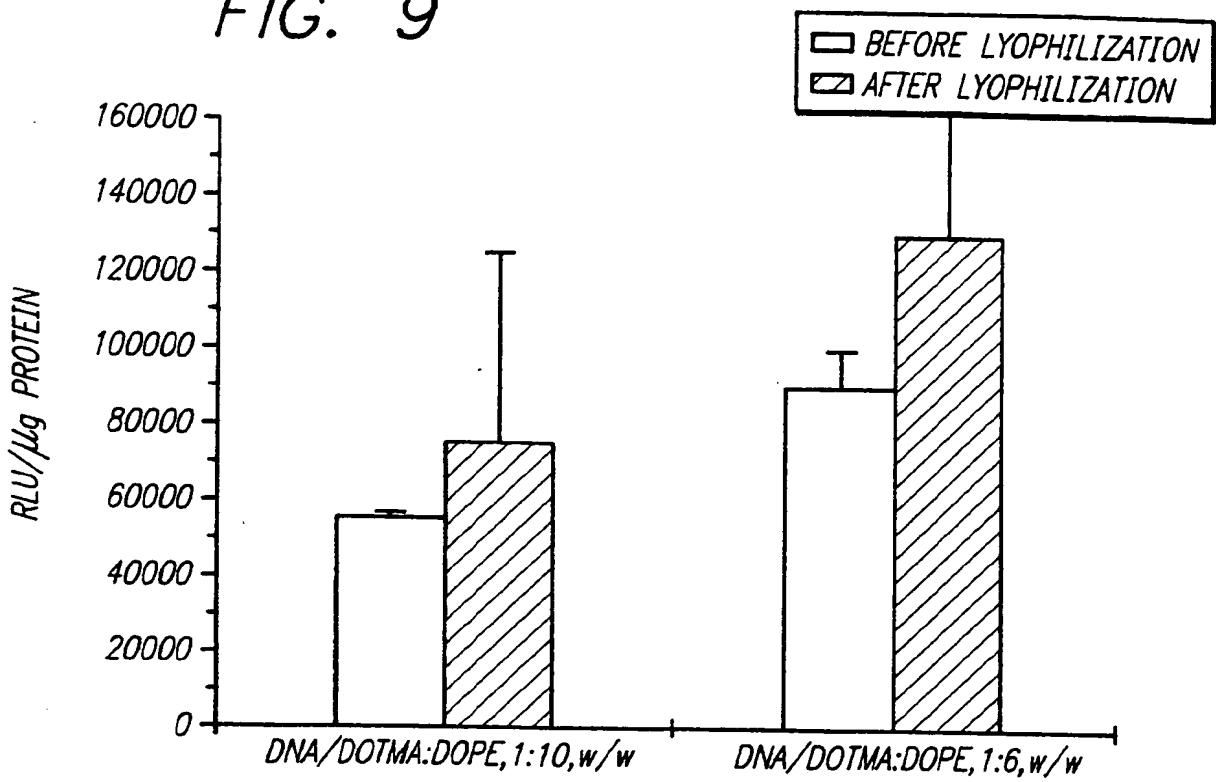


SUBSTITUTE SHEET (RULE 26)

FIG. 8

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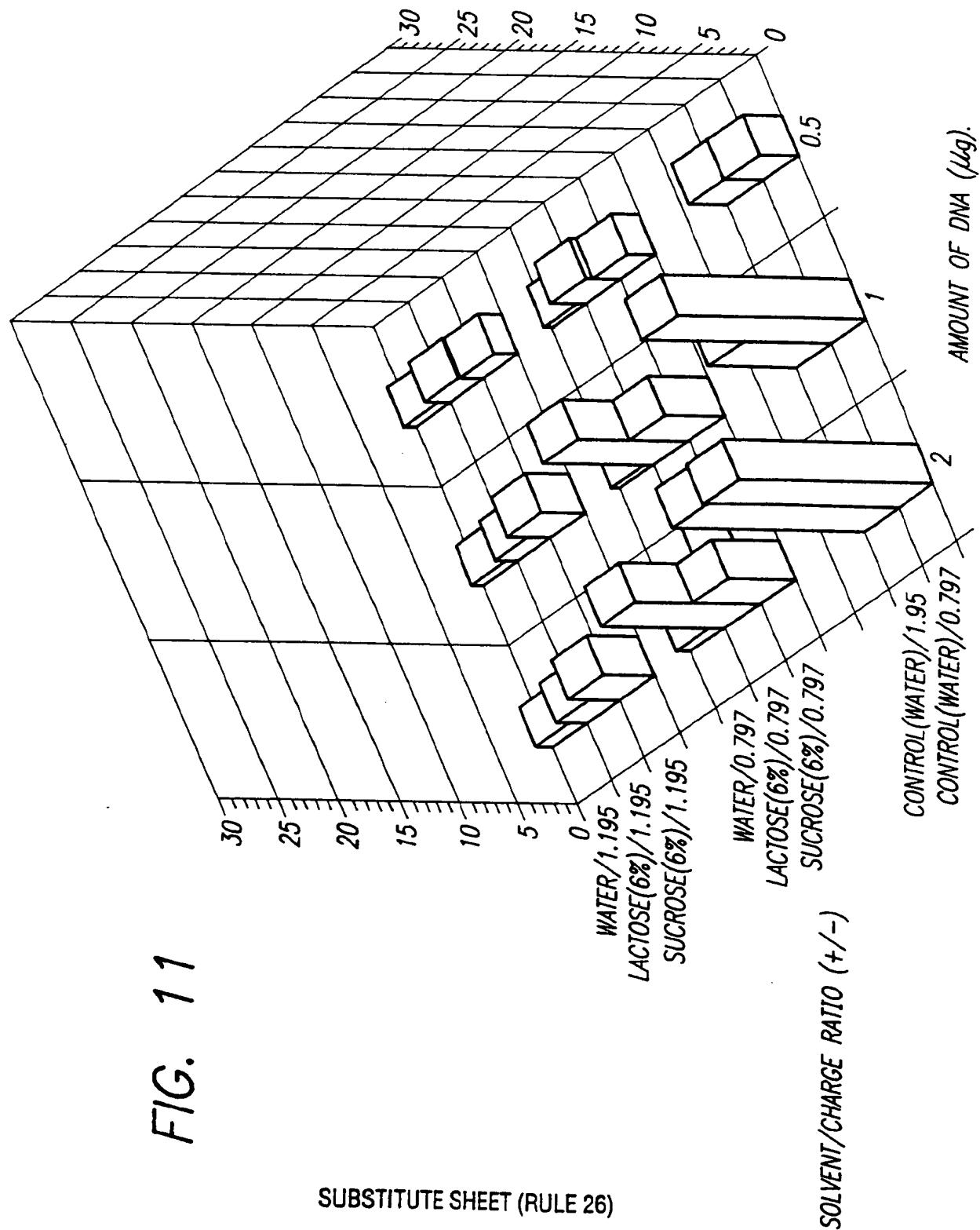
FIG. 9



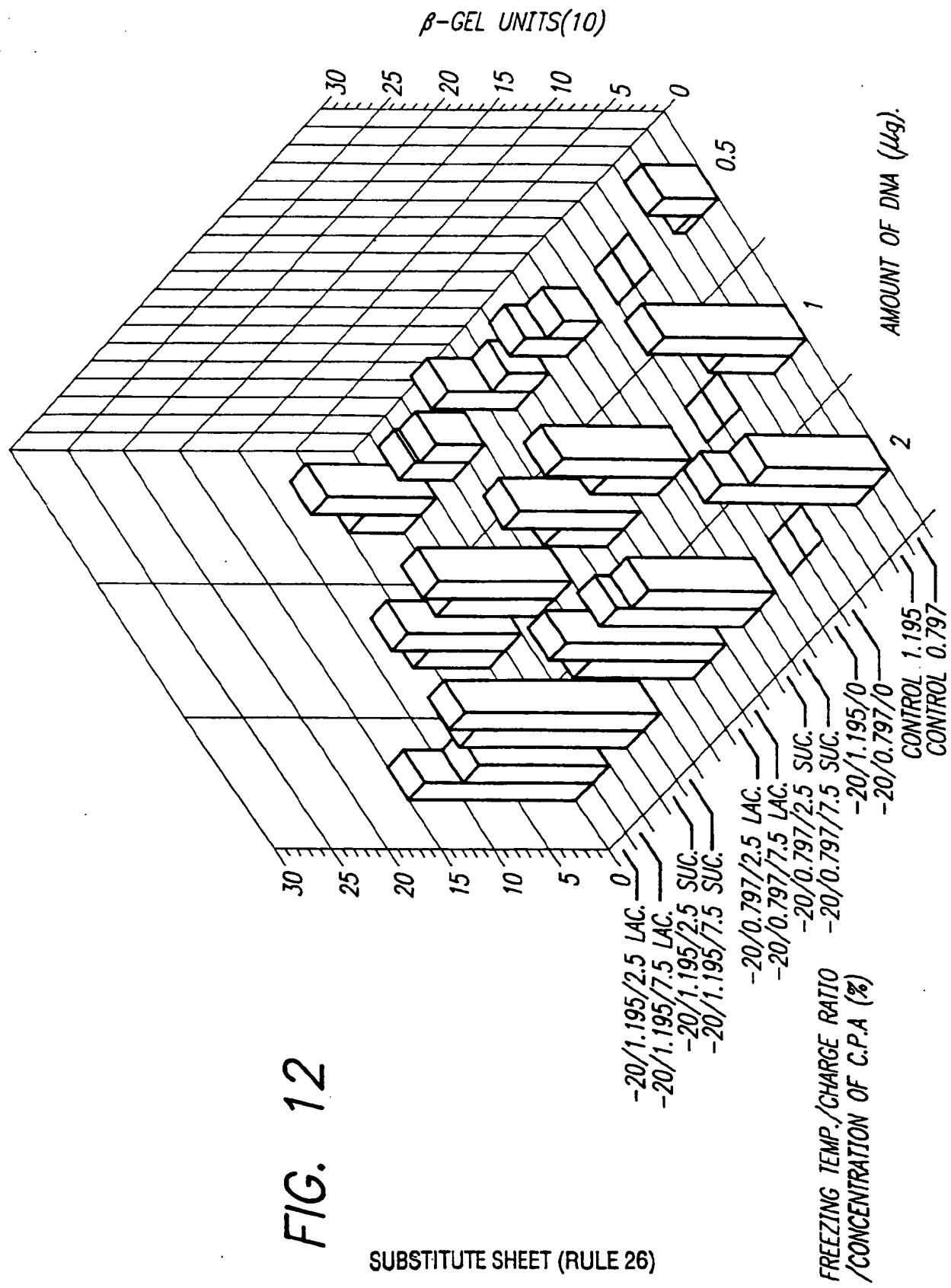
SUBSTITUTE SHEET (RULE 26)

FIG. 10

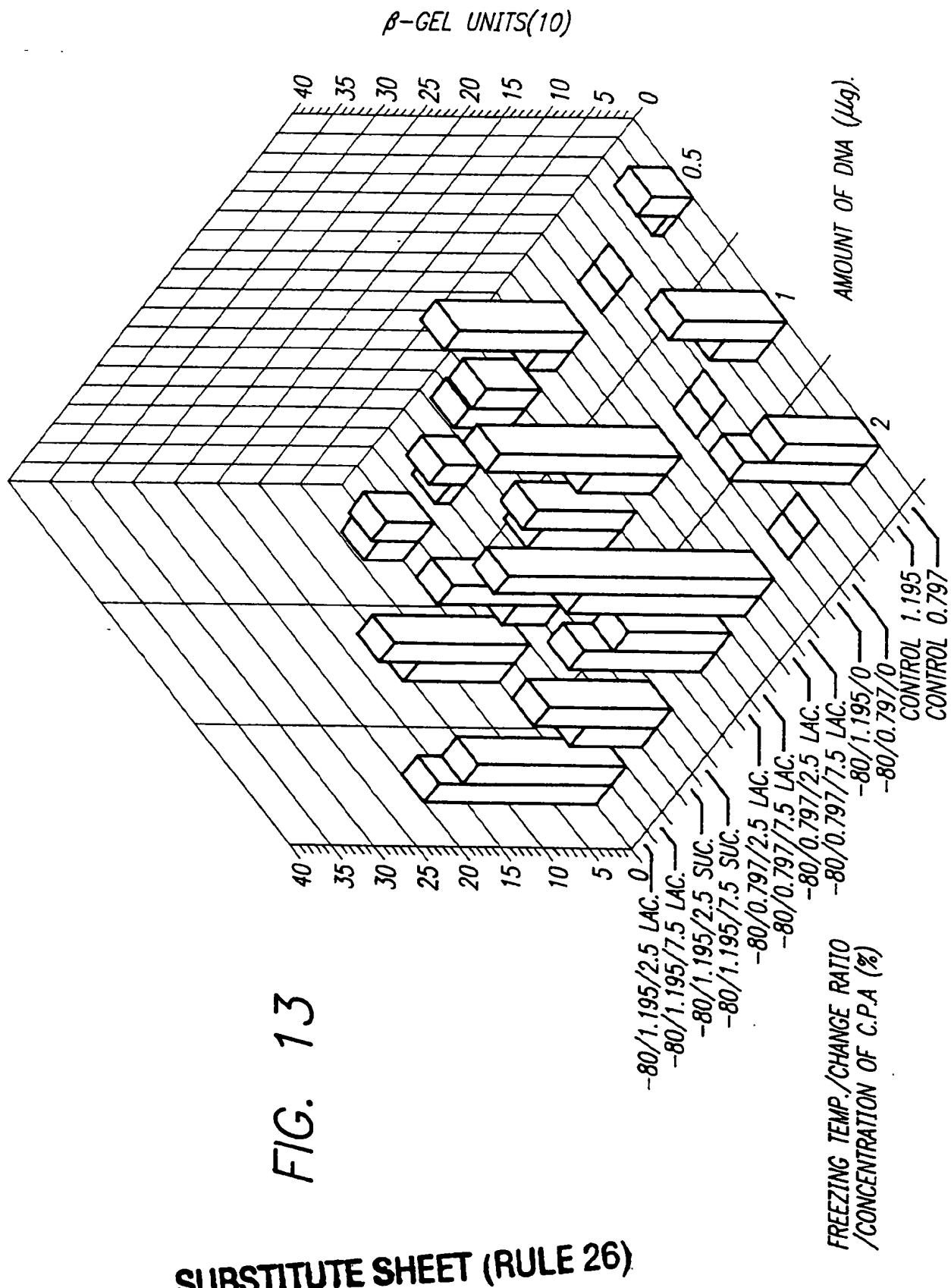
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 $\beta$ -GEL UNITS(10)

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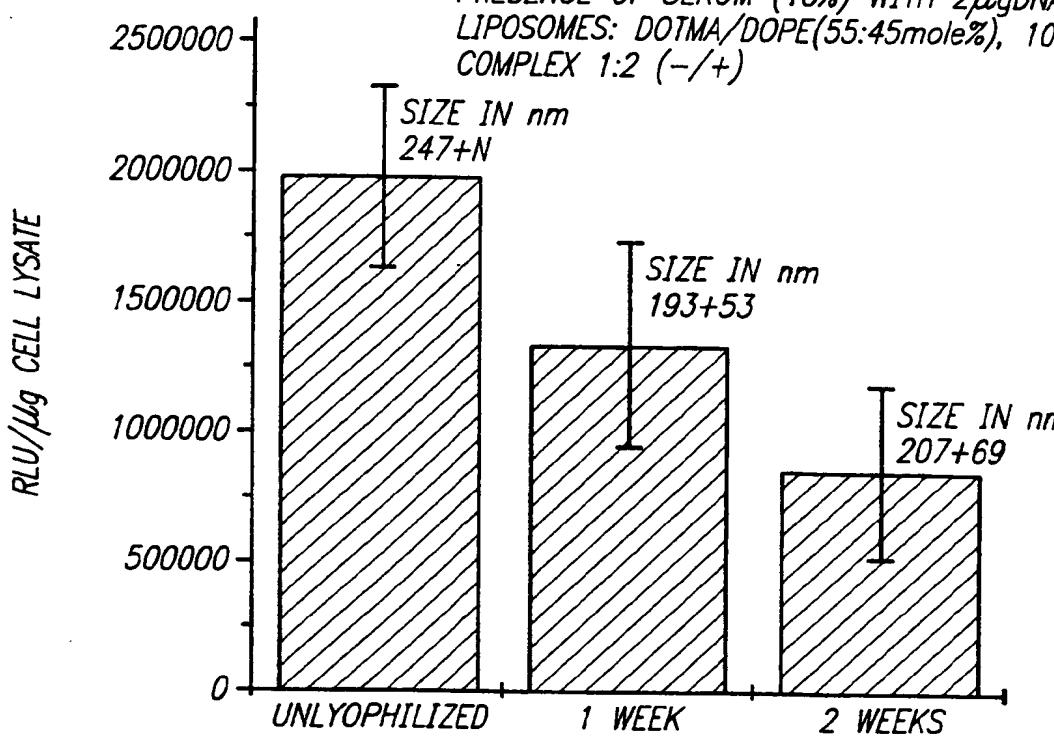
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FIG. 14

CELLS: HIG82 IN 24 WELL PLATE  
 TRANSFECTION IN CONTINUOUS  
 PRESENCE OF SERUM (10%) WITH 2 $\mu$ gDNA/WELL  
 LIPOSOMES: DOTMA/DOPE(55:45mole%), 100nm  
 COMPLEX 1:2 (-/+)



TIME BEFORE LYOPHILIZED, REHYDRATED COMPLEXES USED FOR TRANSFECTION

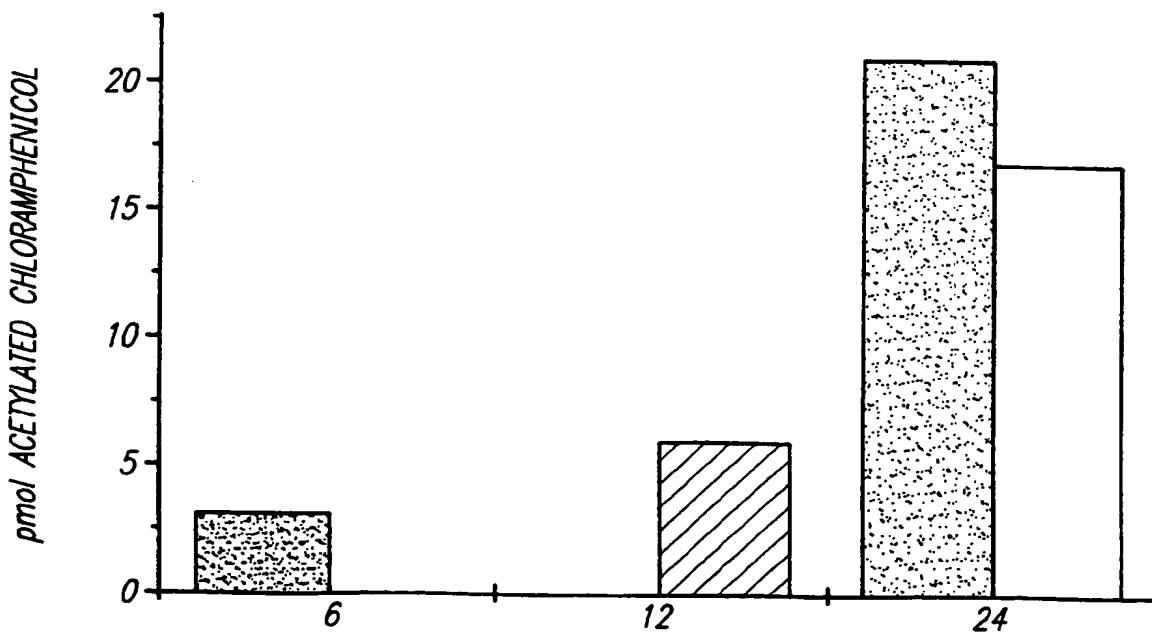
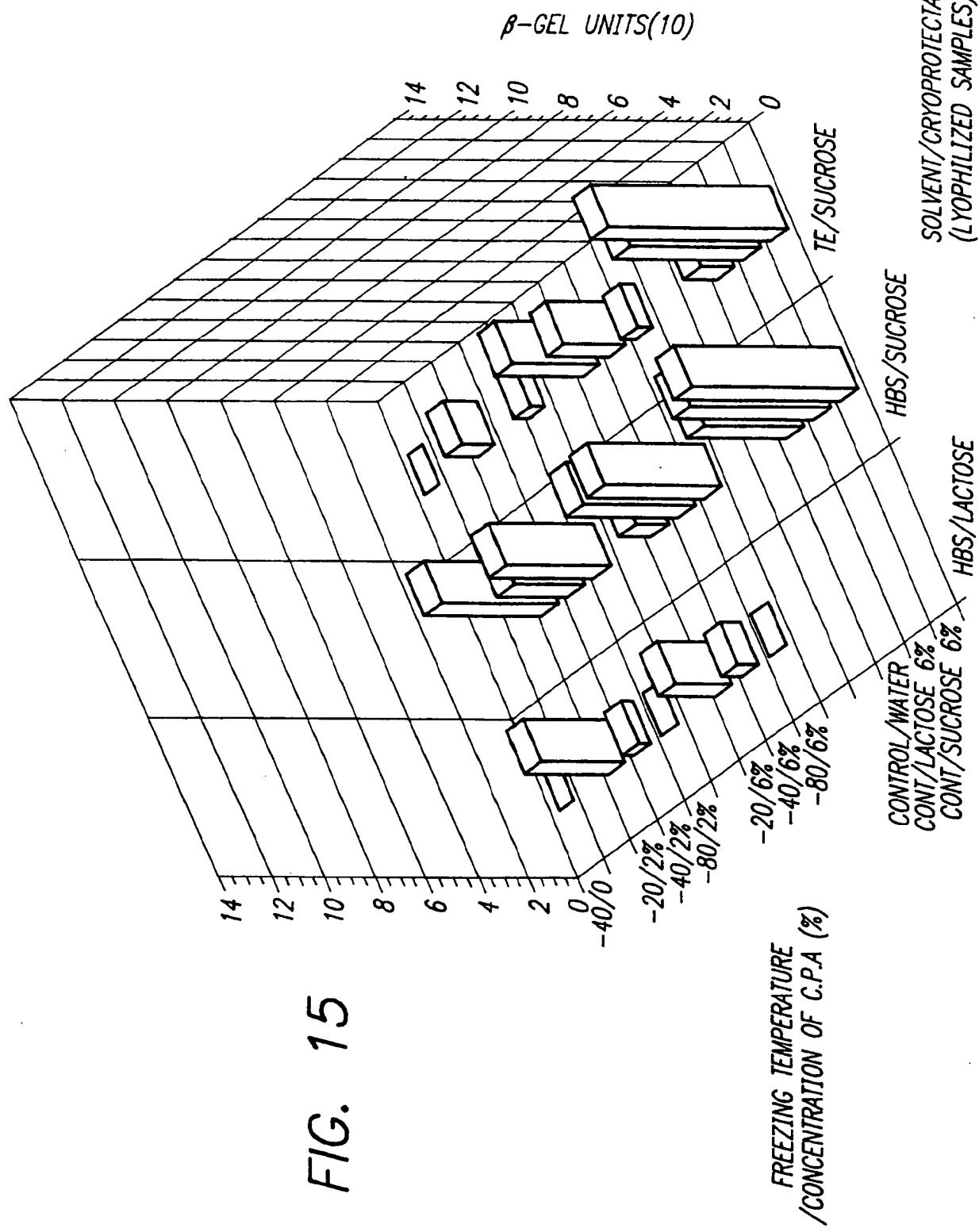


FIG. 18

SUBSTITUTE SHEET (RULE 26)

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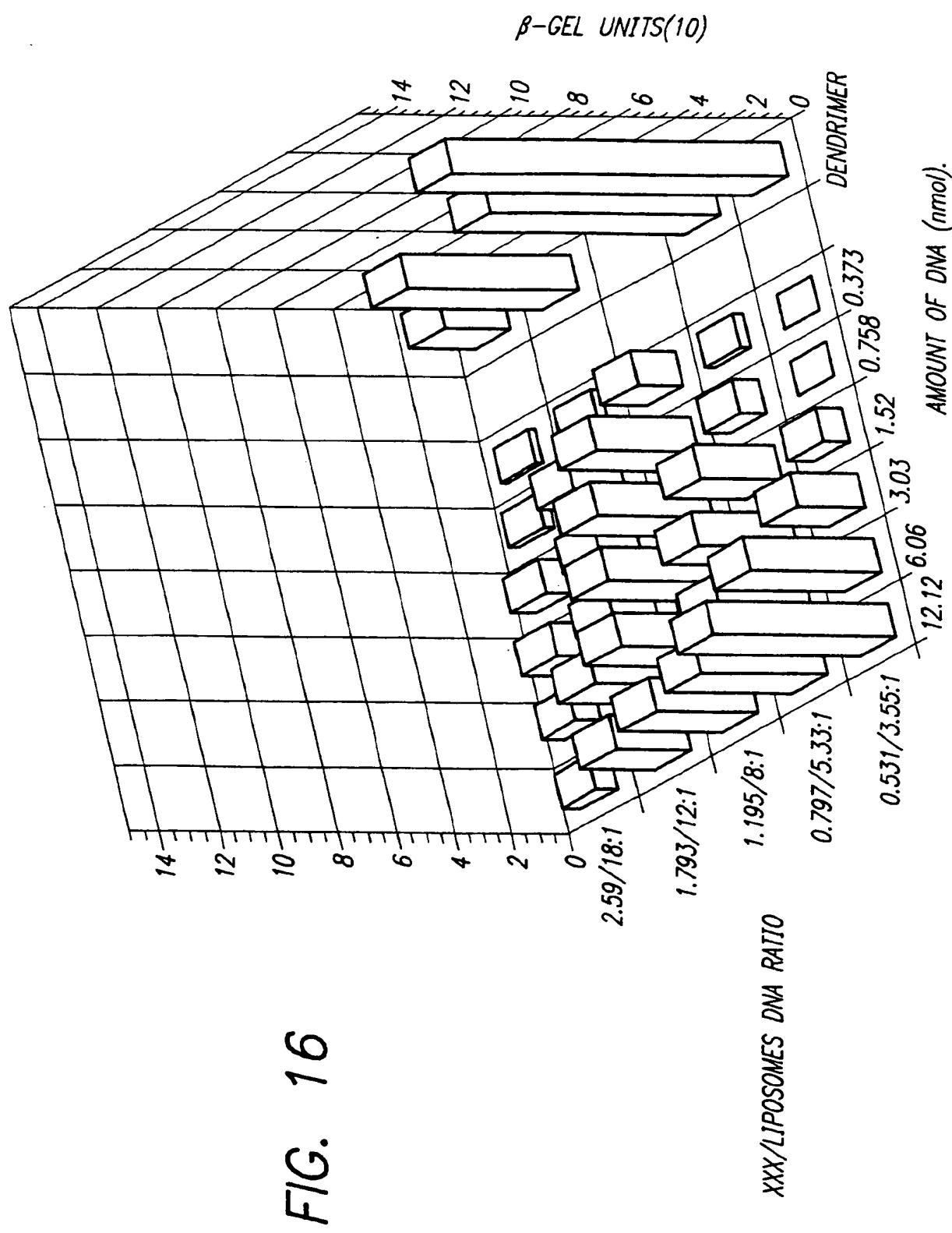
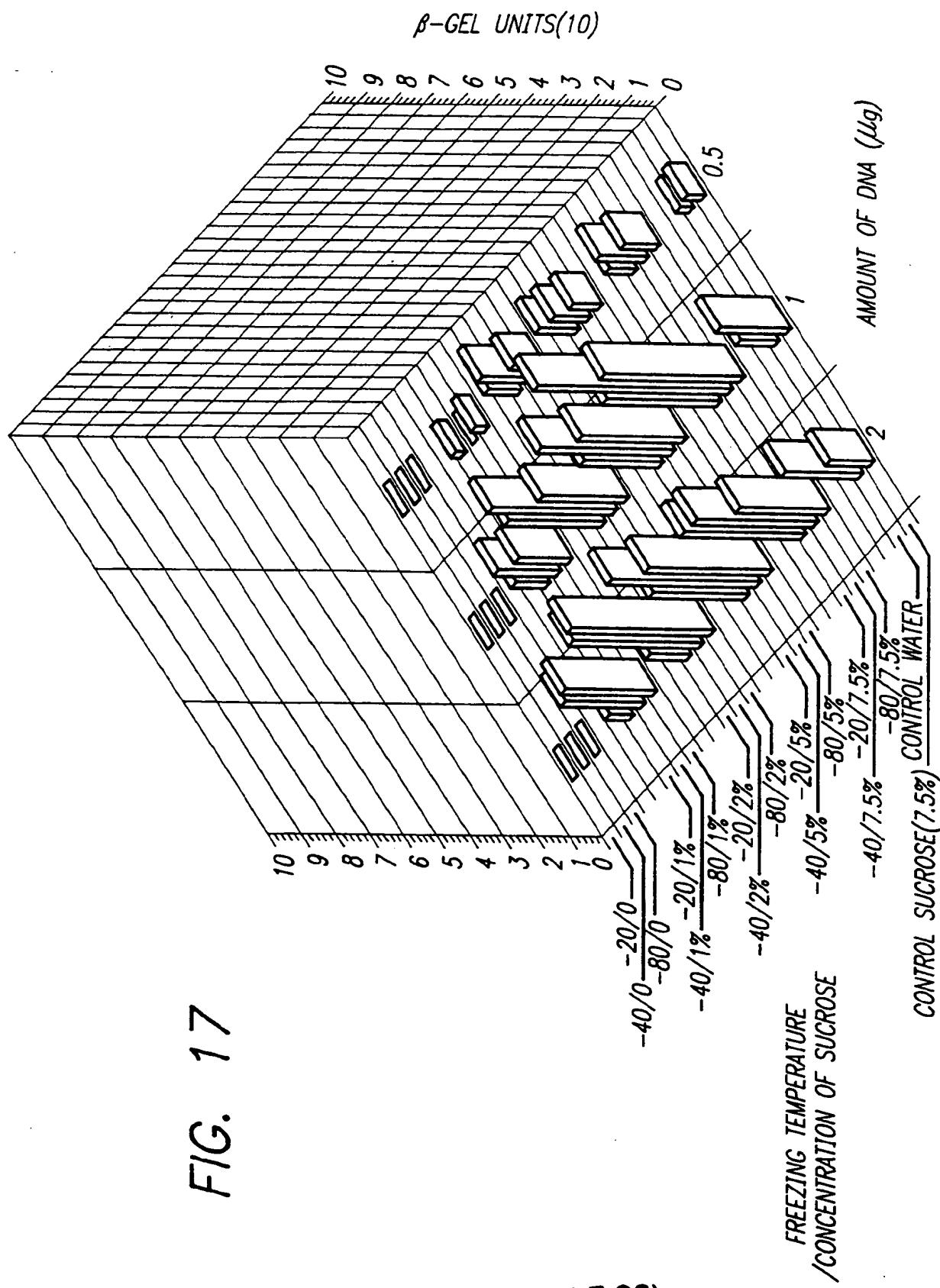


FIG. 16

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FIG. 19

SPERMINE-5-CARBOXYGLYCINE (*N'*-STEARYL - *N'*-OLEYL)  
AMIDE TETRATRIFLUOROACETIC ACID SALT (JK-75)

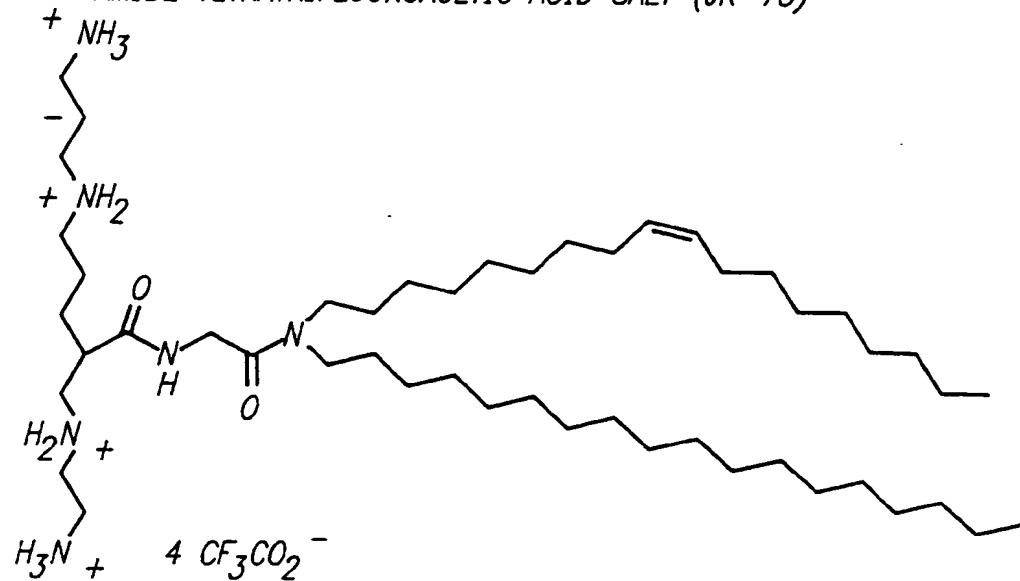
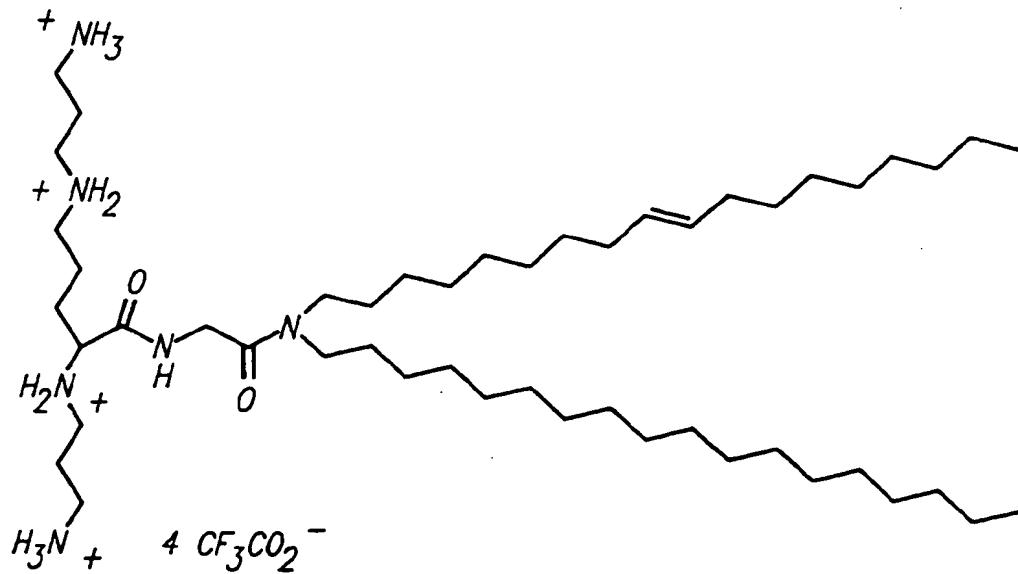


FIG. 20

SPERMINE-5-CARBOXYGLYCINE (*N'*-STEARYL - *N'*-ELAIDYL)  
AMIDE TETRATRIFLUOROACETIC ACID SALT (JK-76)



SUBSTITUTE SHEET (RULE 26)

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## AGMATINYL CARBOXYCHOLESTEROL ACETIC ACID SALT (AG-Chol)

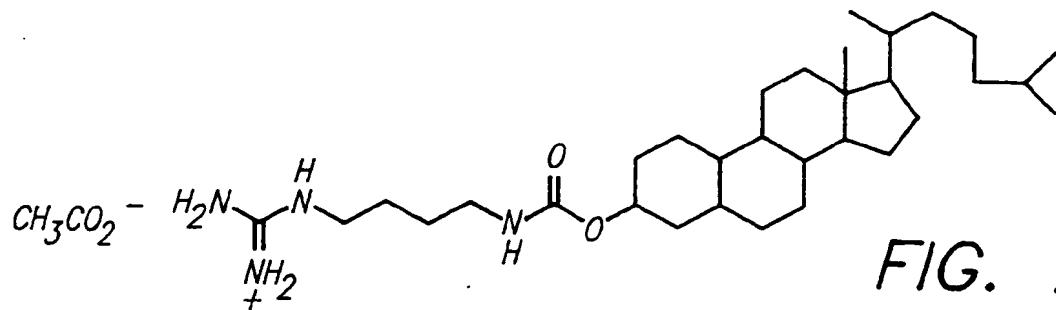


FIG. 21

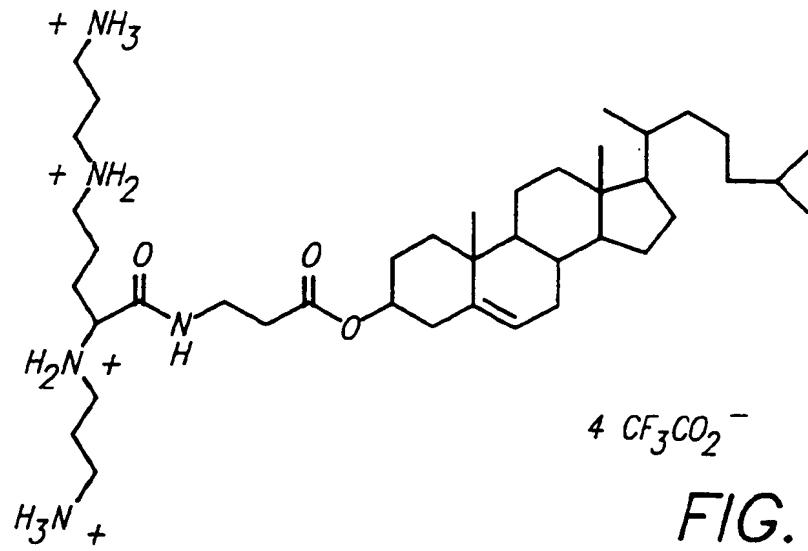
SPERMINE-5-CARBOXY- $\beta$ -ALANINE CHOLESTERYL ESTER TETRATRIFLUOROACETIC ACID SALT (CAS)

FIG. 22

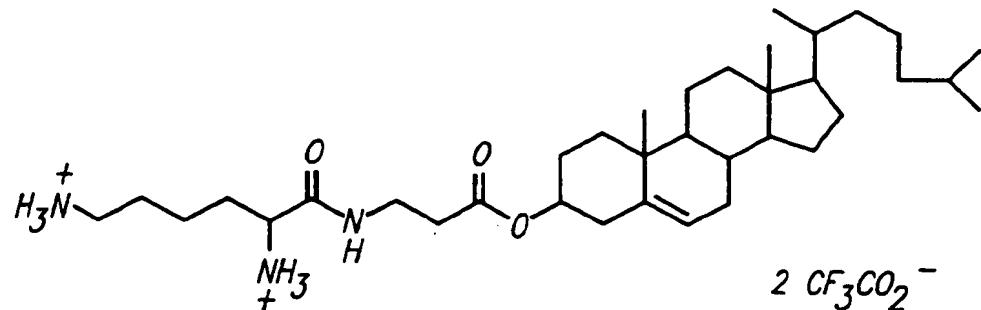
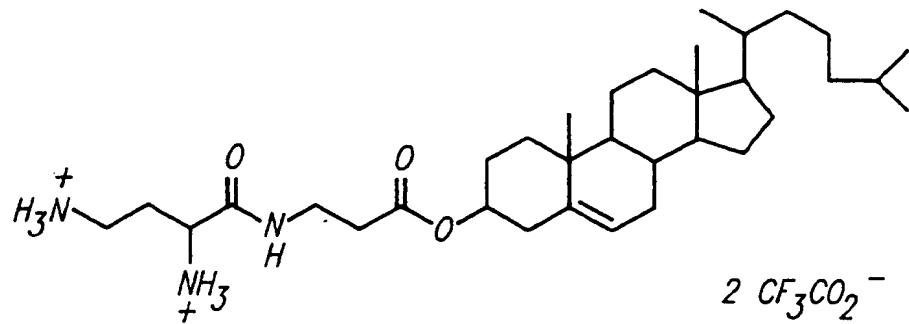
2,6-DIAMINOHEXANOYL  $\beta$ -ALANINE CHOLESTERYL ESTER BISTRIFLUOROACETIC ACID SALT (CAL)

FIG. 23

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FIG. 24

2,4-DIAMINOBUTYROYL  $\beta$ -ALANINE CHOLESTERYL ESTER  
BISTRIFLUOROACETIC ACID SALT (CAB)



*N, N-Bis (3-AMINOPROPYL)-3-AMINOPROPIONYL  $\beta$ -ALANINE CHOLESTERYL ESTER  
TRISTRIFLUOROACETIC ACID SALT (CASD)*

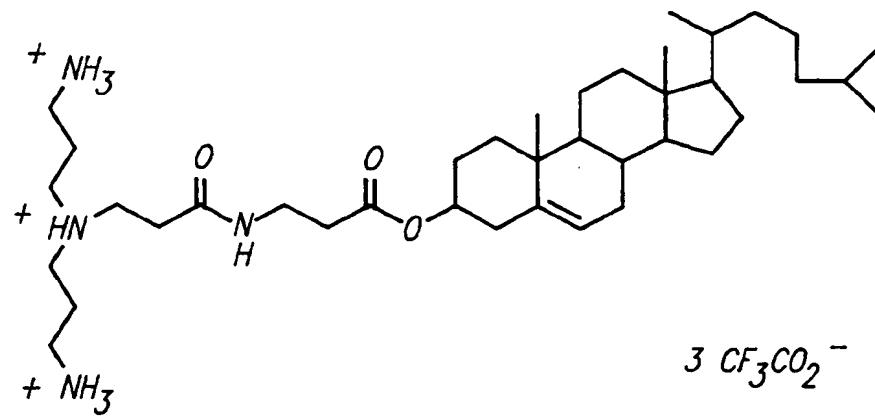


FIG. 25

[*N, N-Bis(2-HYDROXYETHYL)-2-AMINOETHYL]AMINOCARBOXY CHOLESTERYL ESTER  
(JK-154)*

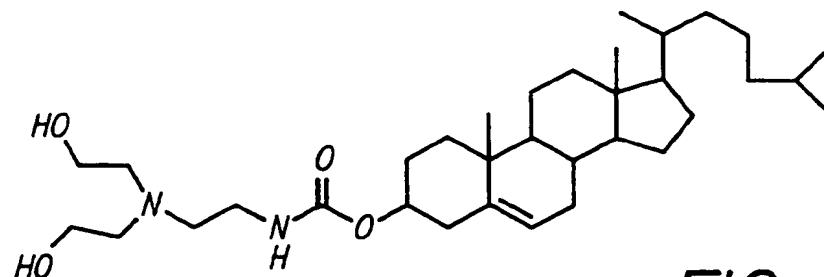


FIG. 26

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## CARNITINE ESTER LIPIDS

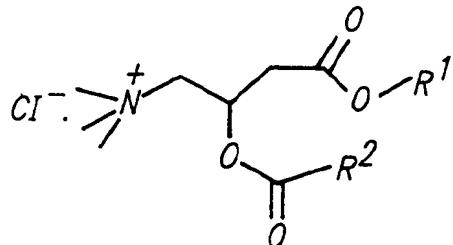
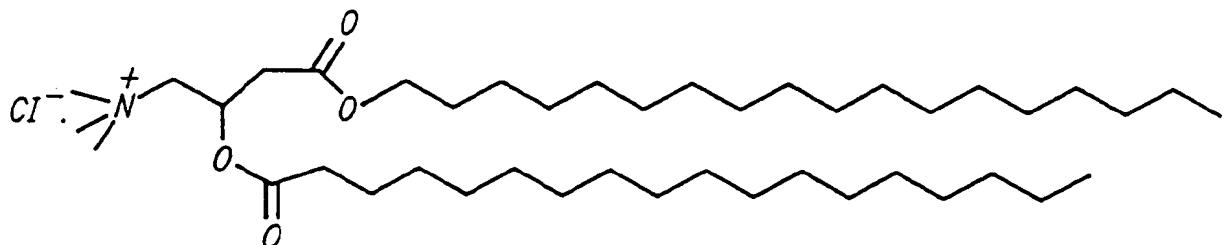


FIG. 27

$R^1 = (CH_2)_n CH_3$   $n = 2$  TO  $30$ ,  $1$  TO  $6$  UNSATURATED BONDS OR ISO  $CH_3$  GROUPS

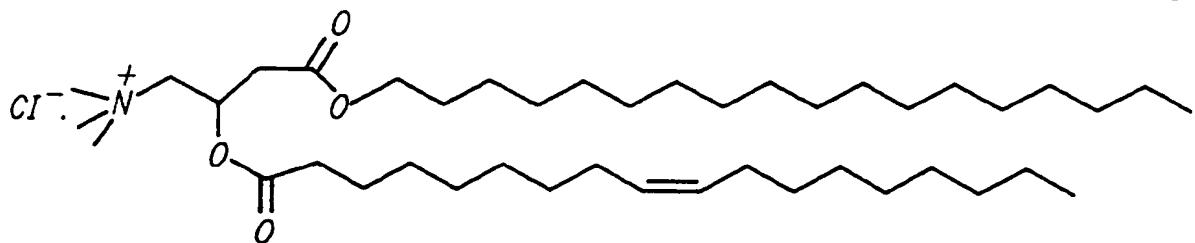
## STEARYL STEAROYL CARNITINE ESTER CHLORIDE SALT (SSCE)

FIG. 28



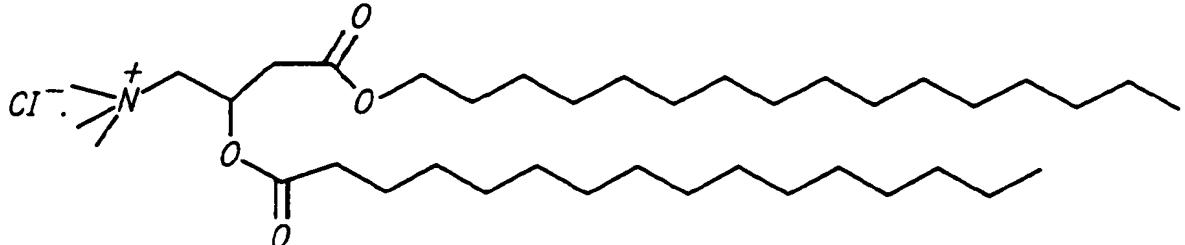
## STEARYL OLEOYL CARNITINE ESTER CHLORIDE (SOCE)

FIG. 29



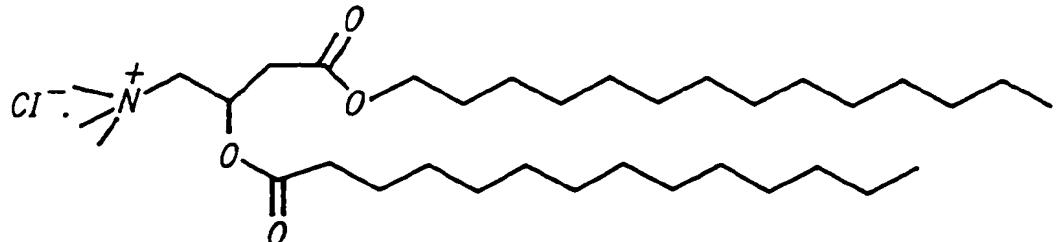
## PALMITYL PALMITOYL CARNITINE ESTER CHLORIDE (PPCE)

FIG. 30

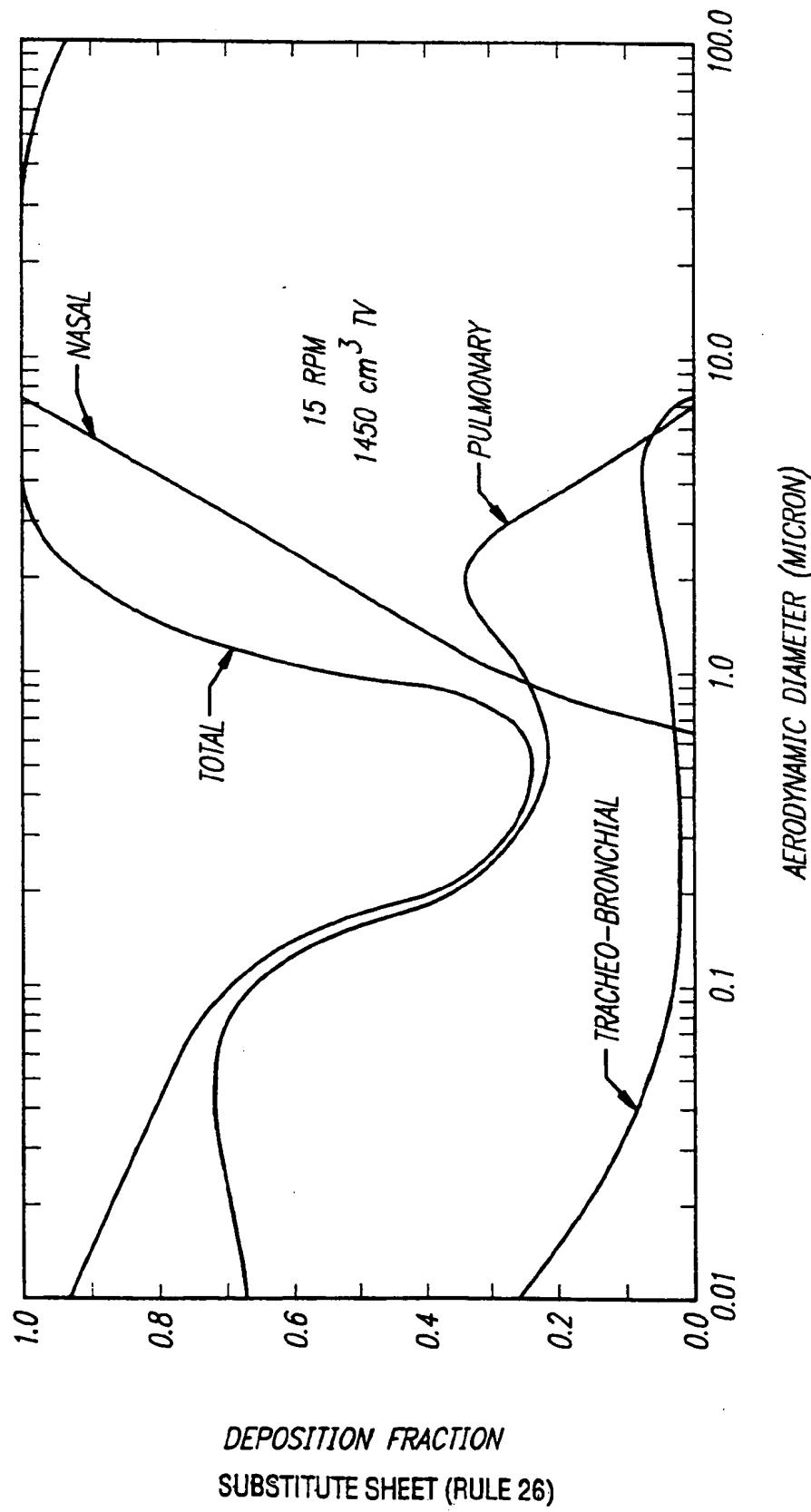


## MYRISTYL MYRISTOYL CARNITINE ESTER CHLORIDE (MMCE)

FIG. 31



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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/07866

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00; C12Q 1/68  
US CL : 435/6; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| Y         | US 5,192,743 A (C.C. HSU) 09 March 1993, column 4, lines 14-29.                      | 1-31                  |
| Y         | US 5,279,833 A (J.K. ROSE) 18 January 1994, column 2, line 38 to column 3, line 29.  | 1-31                  |
| Y         | US 5,334,761 A (G. GEBEYEHU) 02 August 1994, column 3, line 31 to column 4, line 60. | 6-10                  |
| A,E       | US 5,460,831 A (N. KOSSOVSKY) 24 October 1995, column 4, lines 16-47.                | 1-31                  |
| A,E       | US 5,521,291 A (D.T. CURIEL) 28 May 1996, column 3, line 61 to column 4, line 26.    | 1-31                  |

 Further documents are listed in the continuation of Box C.  See patent family annex.

|   |     |  |
|---|-----|--|
| • Special categories of cited documents:  | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| "A" document defining the general state of the art which is not considered to be of particular relevance  | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| "E" earlier document published on or after the international filing date  | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family  |
| "O" document referring to an oral disclosure, use, exhibition or other means  |     |  |
| "P" document published prior to the international filing date but later than the priority date claimed  |     |  |

Date of the actual completion of the international search

18 AUGUST 1996

Date of mailing of the international search report

16 SEP 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231Authorized officer  
THANDA WAI  
Signature

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/07866

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |  |                       |
|---|--|-----------------------|
| Category*   | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
| A,P   | KUKOWSKA-LATALLO et al. Efficient Transfer of Genetic Material into Mammalian Cells Using Starburst Polyamidoamine Dendrimers. Proceedings of the National Academy of Sciences USA. 14 May 1996, Vol. 93, pages 4897-4902, especially page 4897, abstract. | 6                     |

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US96/07866

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US96/07866

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, CAPLUS, WPIDS

search terms: polynucleotide, cryoprotection, lyophilization, transfection, transformation, delivery, gene therapy

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

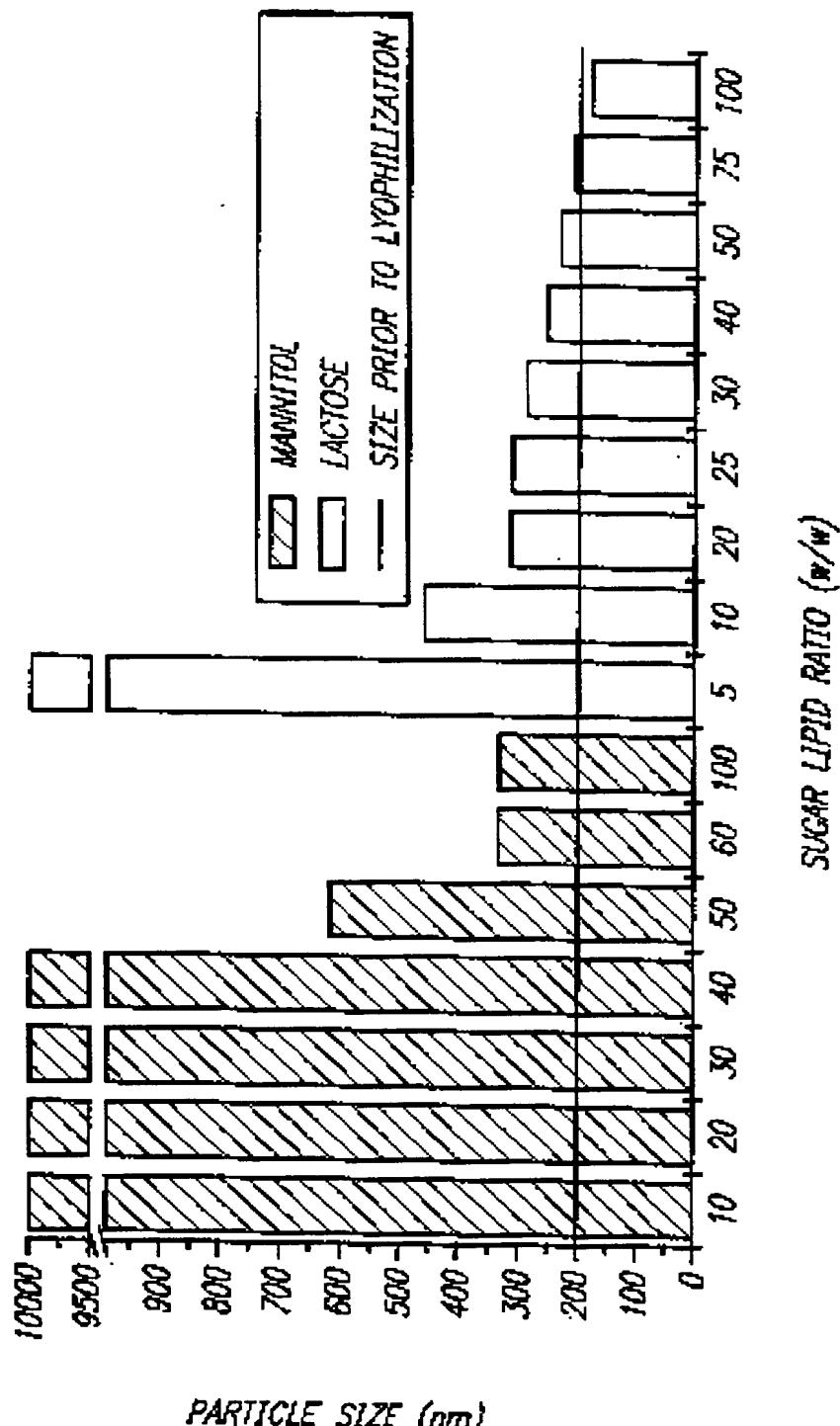
This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-24, drawn to a composition comprising a lyophilized formulation of a polynucleotide complex and a cryoprotectant (first product) and to method of treating a polynucleotide using the composition.

Group II, claims 25-31, drawn to a method for gene therapy using the composition of Group I (second method of using).

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the composition of Group I is used in two distinct methods, the first to treat a polynucleotide, and the second to use the composition for gene therapy. PCT Rule 13 does not provide for multiple methods of using within a single application (37 CFR 1.475(d)).

FIG. 1

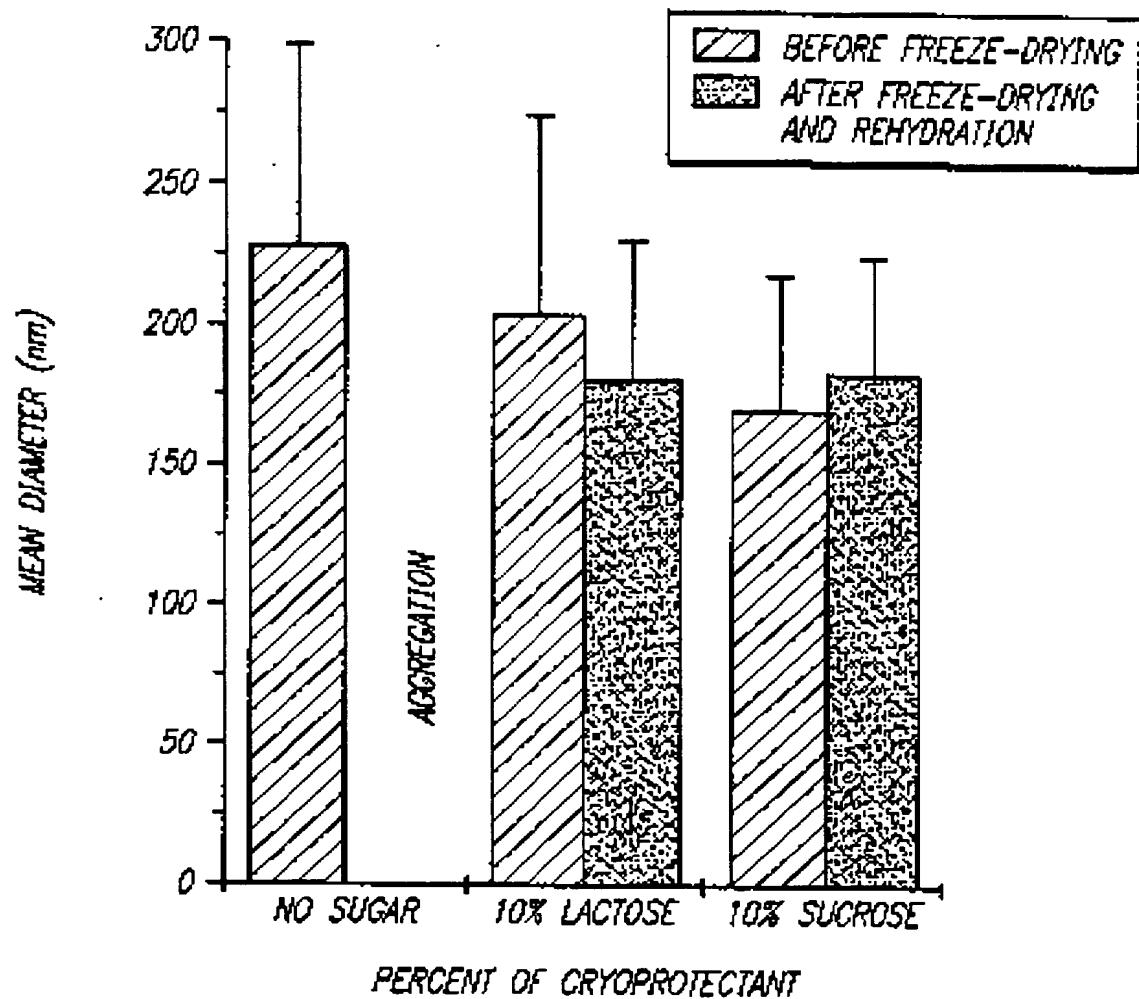


PARTICLE SIZE (nm)

SUBSTITUTE SHEET (RULE 26)

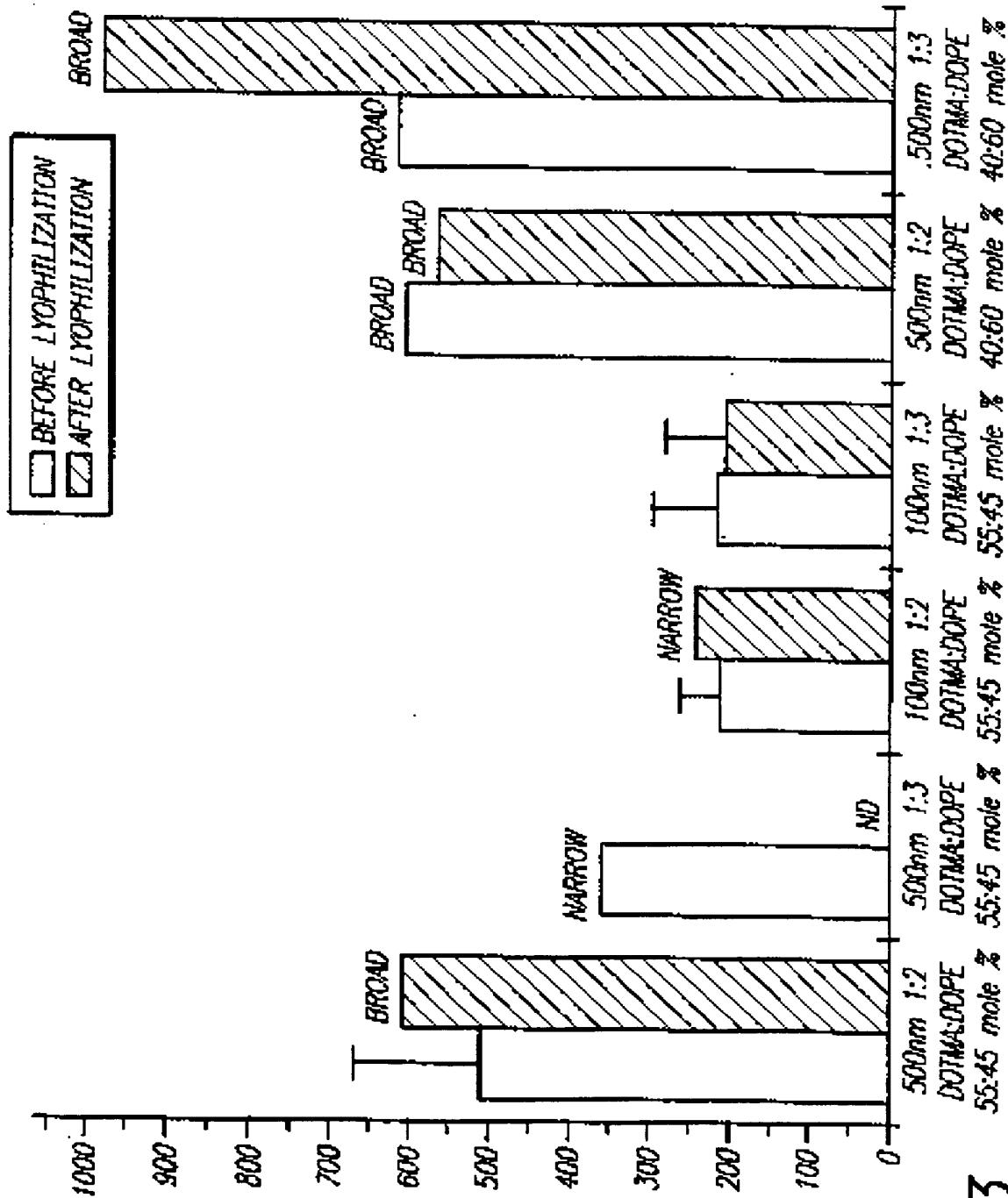
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FIG. 2



- DNA/DOTMA:DOPE RATIO = 1:10 w/w
- LIPOSOME:SUGAR RATIO = 1:100 w/w
- DNA CONCENTRATION = 100 μg/mL
- SIMILAR RESULTS WERE OBTAINED WITH 5% CRYOPROTECTANT.

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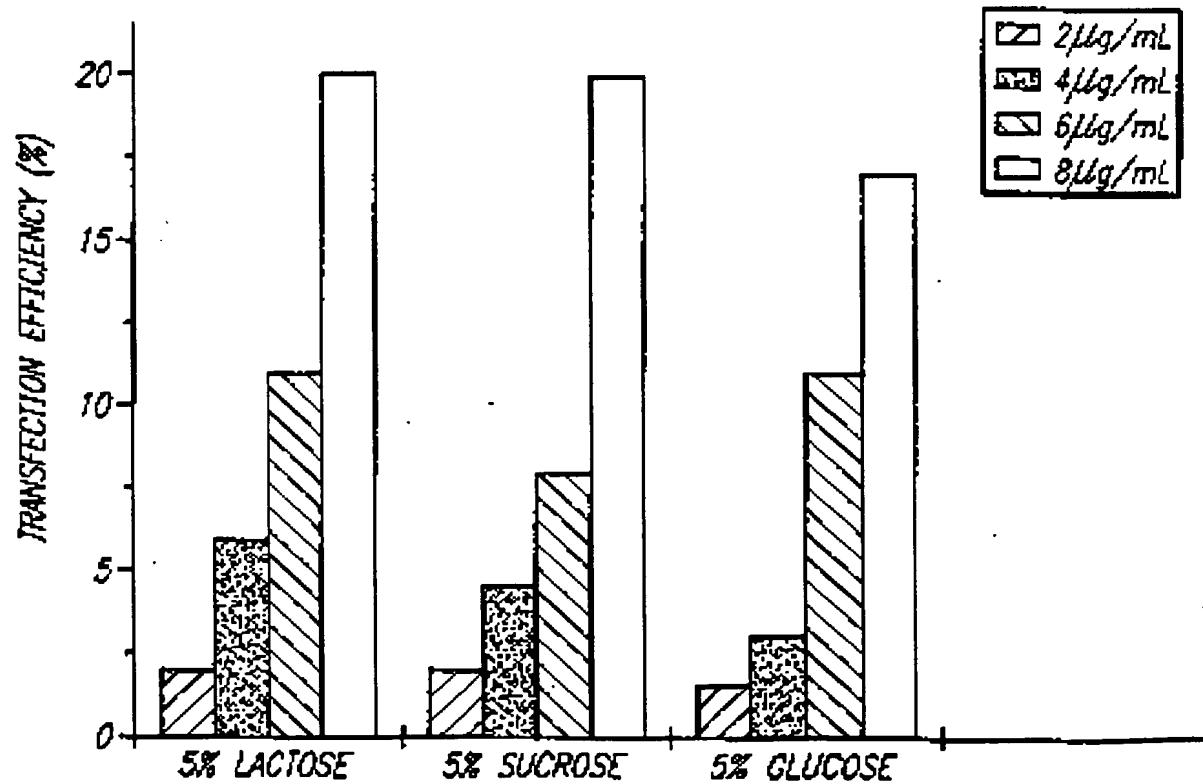
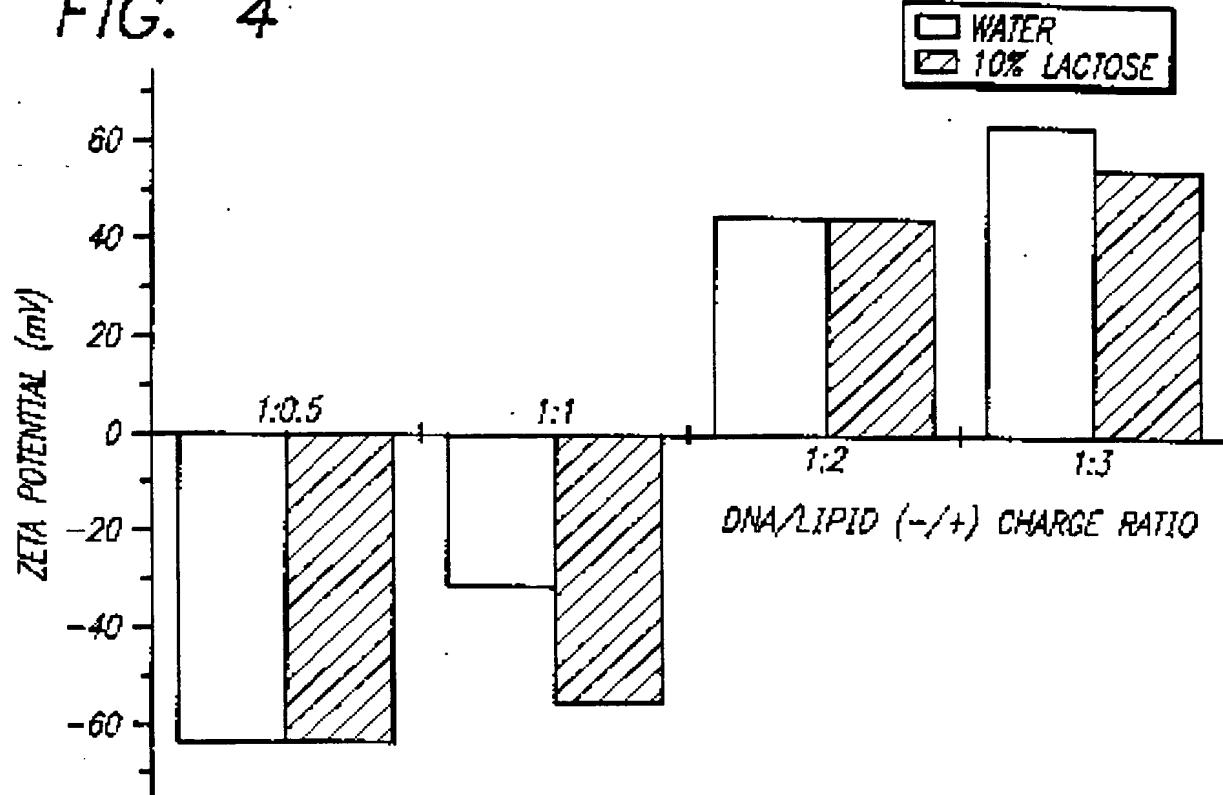
FORMULATION: -/+ CHARGE RATIO, SIZE OF LIPOSOMES

FIG. 3

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FIG. 4



SUBSTITUTE SHEET (RULE 26)

FIG. 6

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1% AGAROSE GEL ELECTROPHORESIS OF DNA/LIPID COMPLEX  
IN THE PRESENCE OF 10% LACTOSE:

BEFORE LYOPHILIZATION

LANE 1: 1:2 -/+ , DOTMA:DOPE, 55:45 mol%, 100nm  
LANE 2: 1:2 -/+ , DOTMA:DOPE, 40:60 mol%, 100nm  
LANE 3: 1:3 -/+ , DOTMA:DOPE, 40:60 mol%, 100nm  
LANE 4: 1:0.5 -/+ , DOTMA:DOPE, 55:45 mol%, 100nm  
LANE 5: FREE DNA  
LANE 6: MOLECULAR STANDARD

AFTER LYOPHILIZATION AND REHYDRATION

LANE 7: 1:2 -/+ , DOTMA:DOPE, 55:45 mol%, 100nm  
LANE 8: 1:3 -/+ , DOTMA:DOPE, 40:60 mol%, 100nm  
LANE 9: 1:3 -/+ , DOTMA:DOPE, 40:60 mol%, 100nm  
LANE 10: 1:0.5 -/+ , DOTMA:DOPE, 55:45 mol%, 100nm

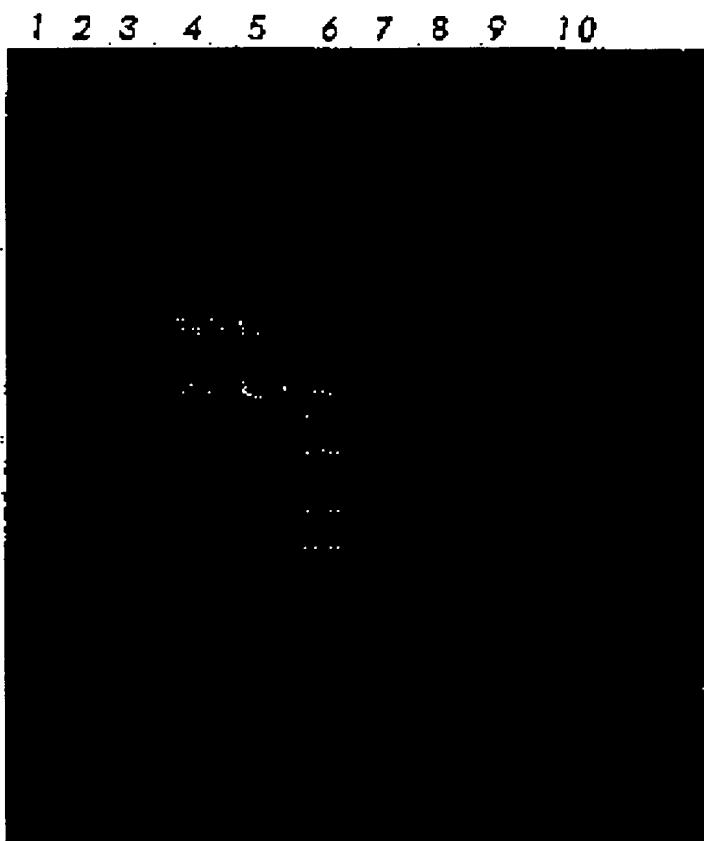
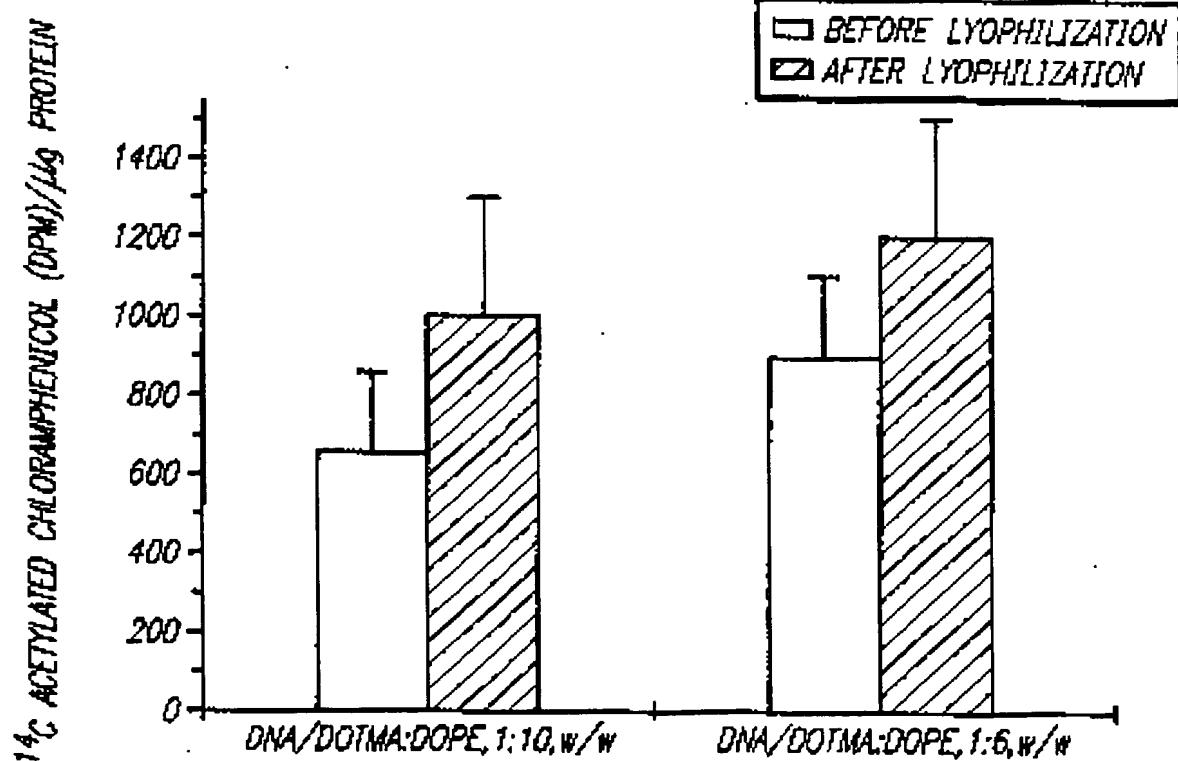
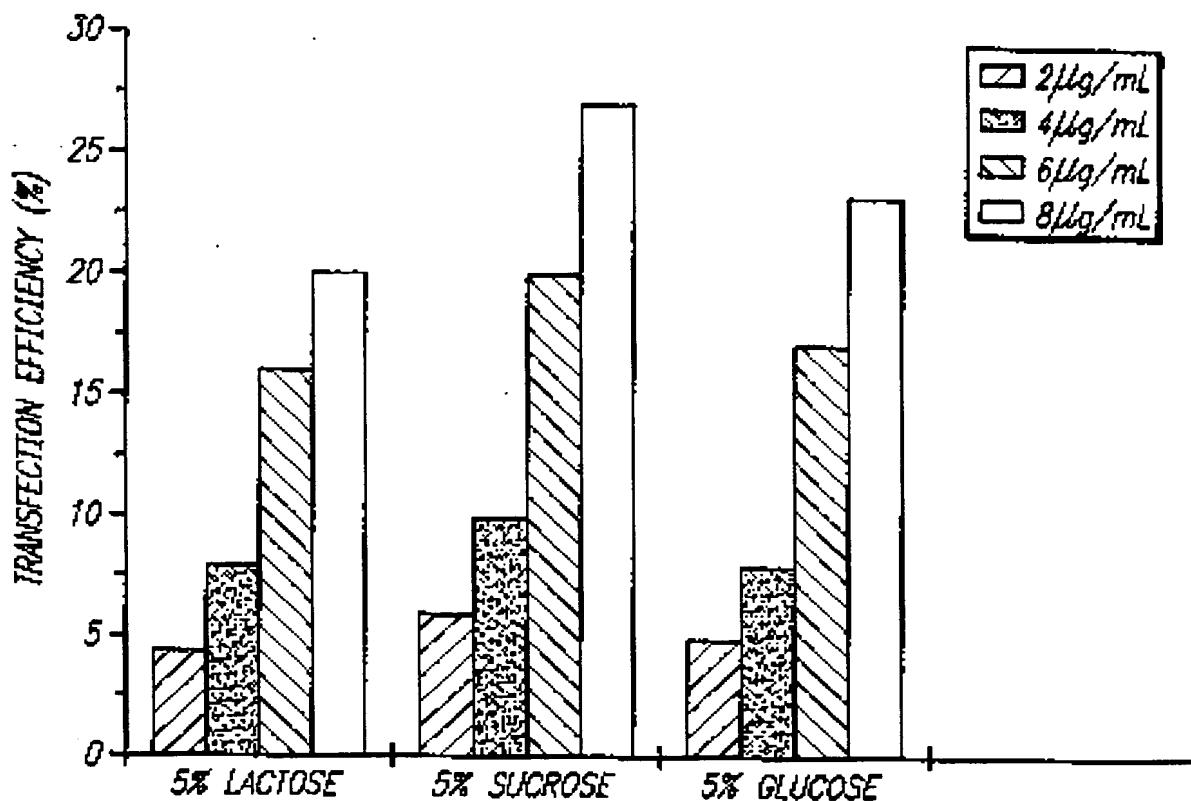


FIG. 5

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FIG. 7



SUBSTITUTE SHEET (RULE 26)

FIG. 8

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FIG. 9

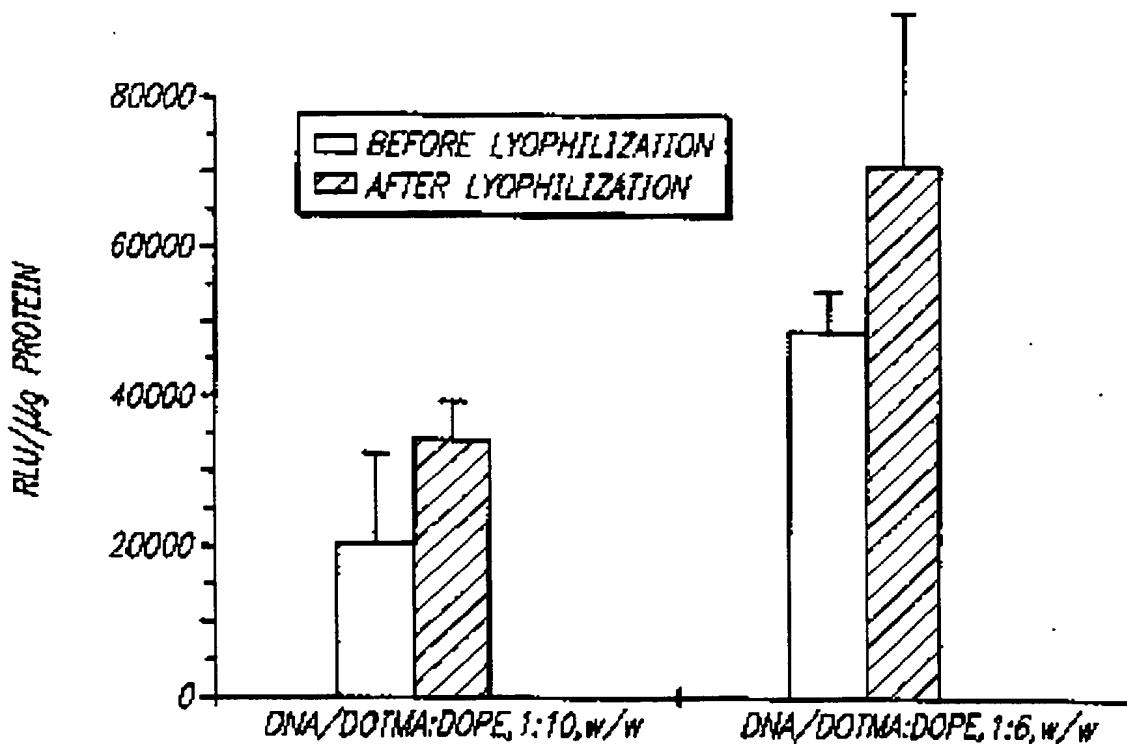
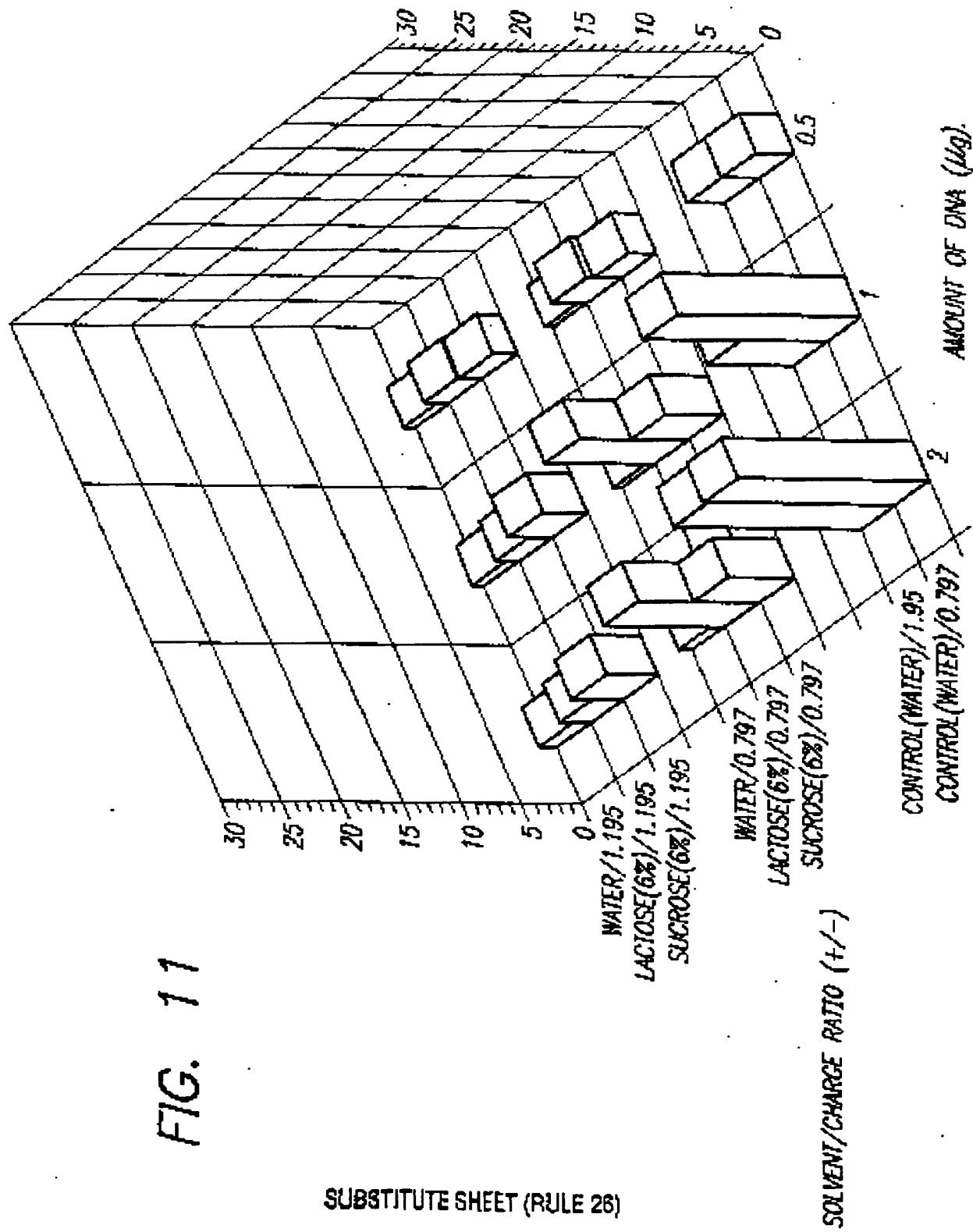


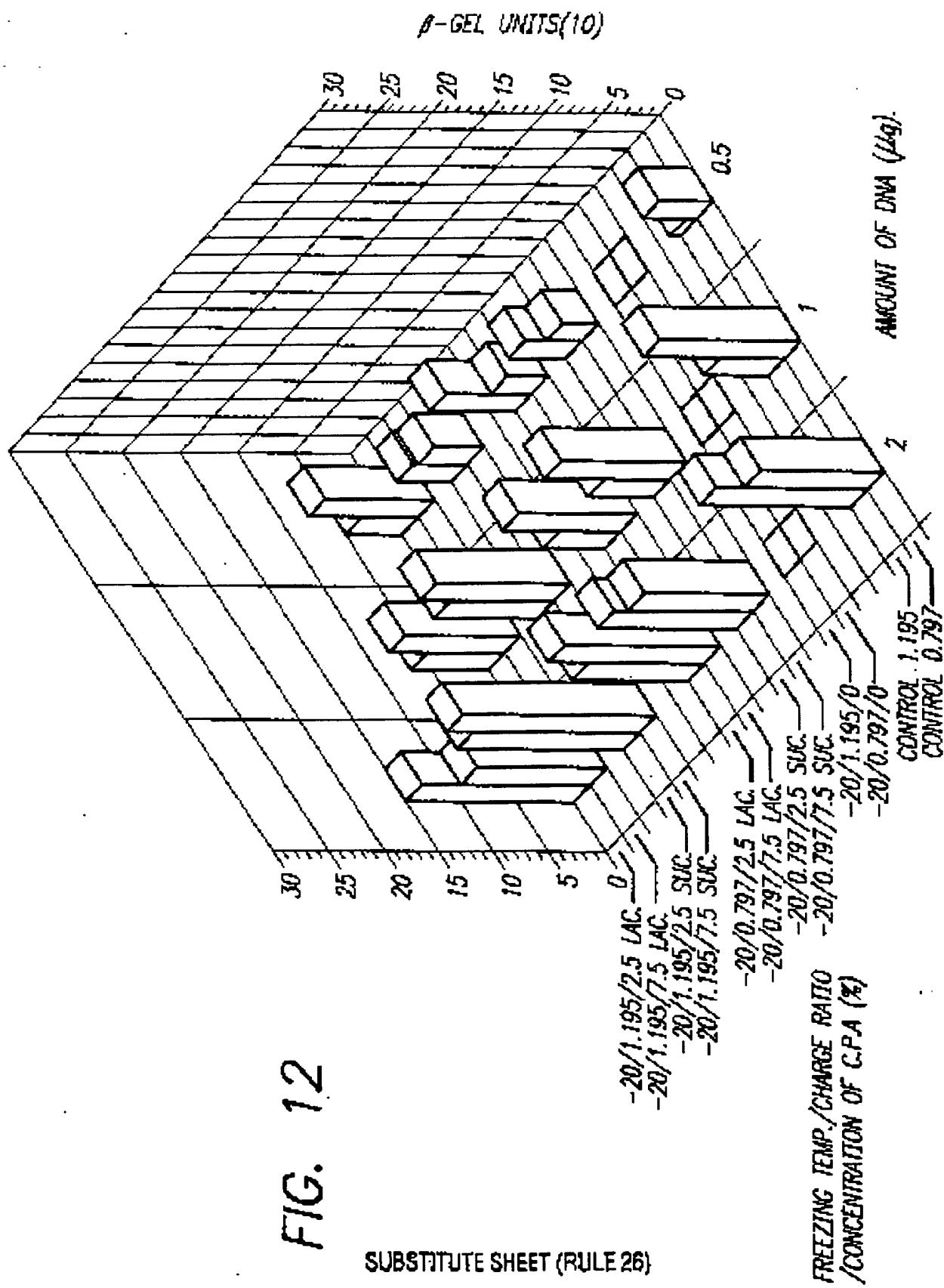
FIG. 10

SUBSTITUTE SHEET (RULE 26)

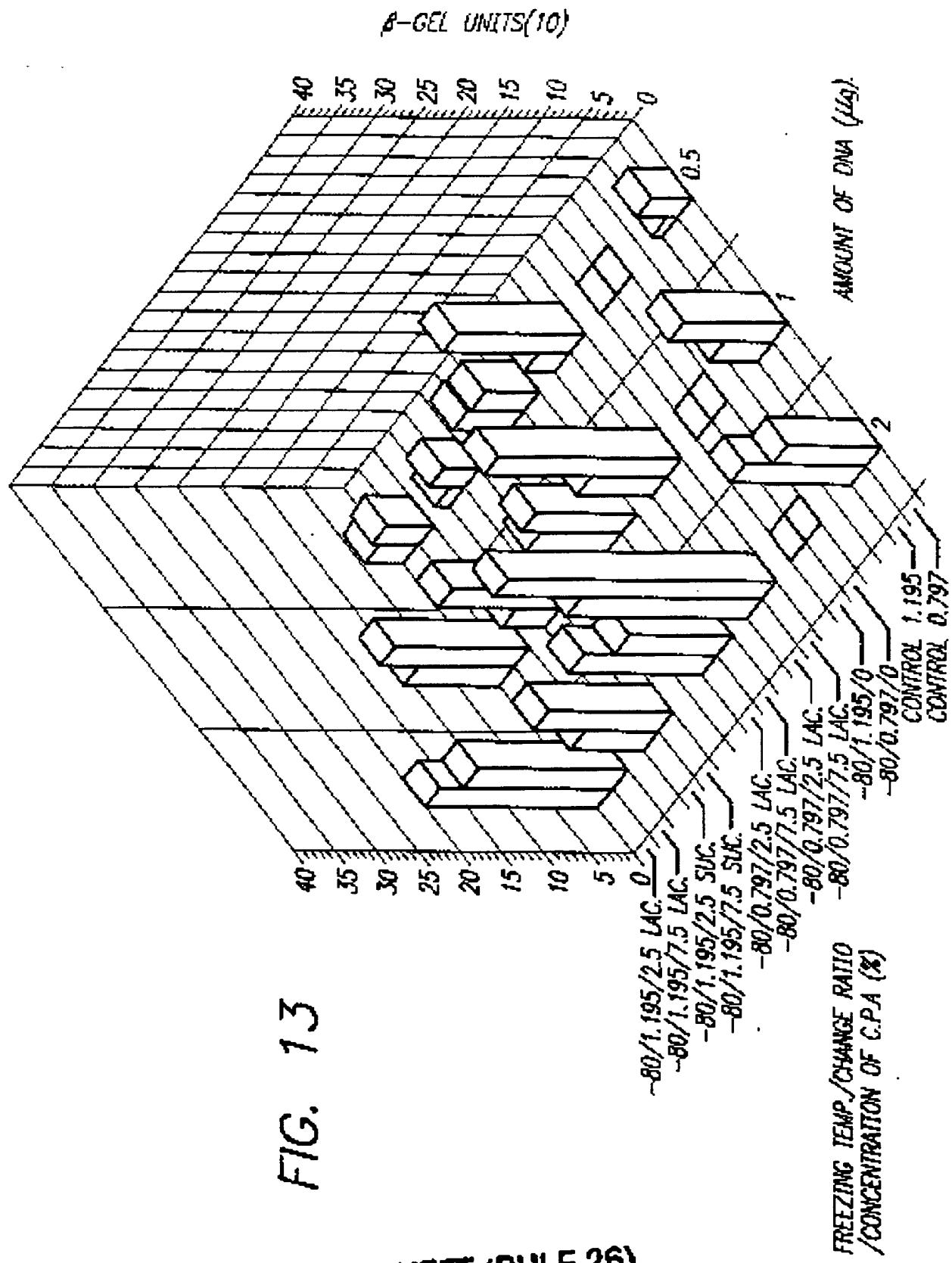
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 $\beta$ -GEL UNITS(10)

9/19



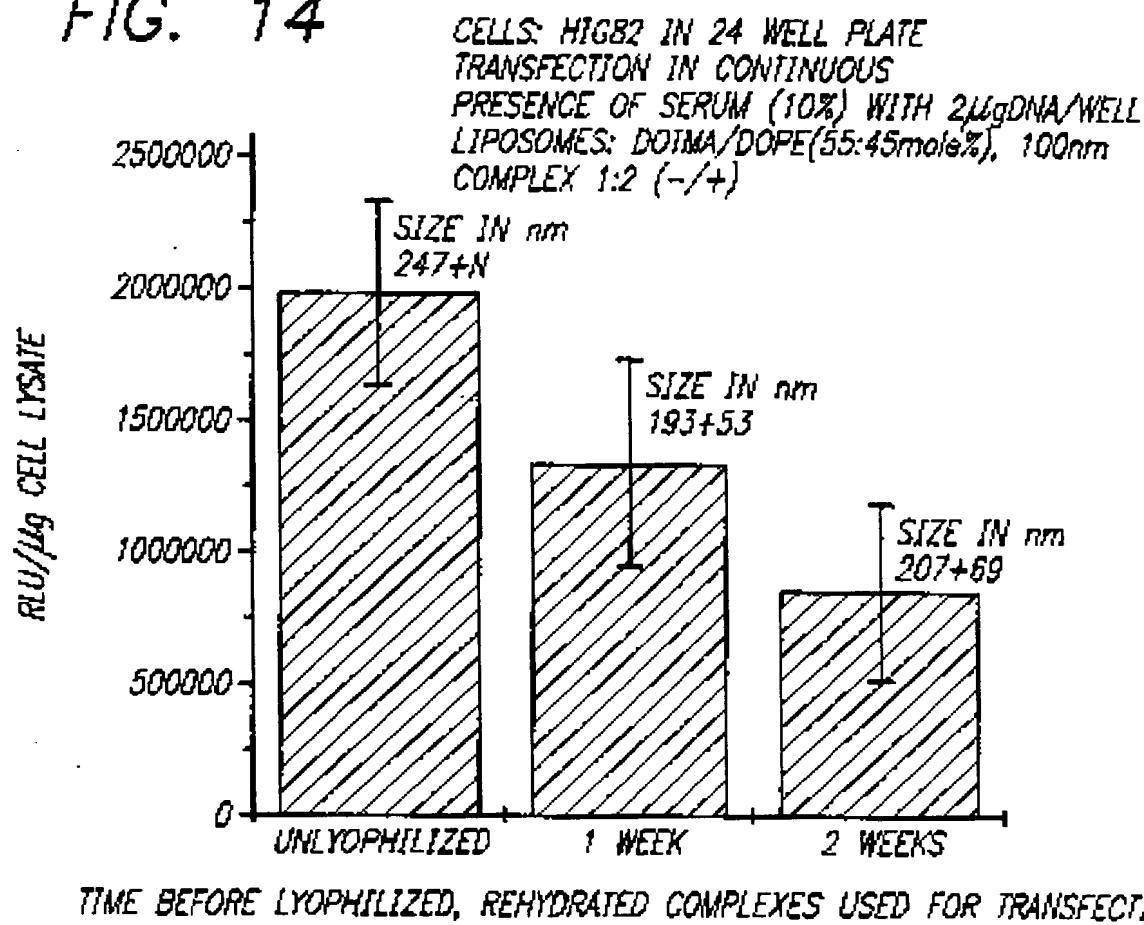
10/19



**SUBSTITUTE SHEET (RULE 26)**

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FIG. 14



TIME BEFORE LYOPHILIZED, REHYDRATED COMPLEXES USED FOR TRANSFECTION

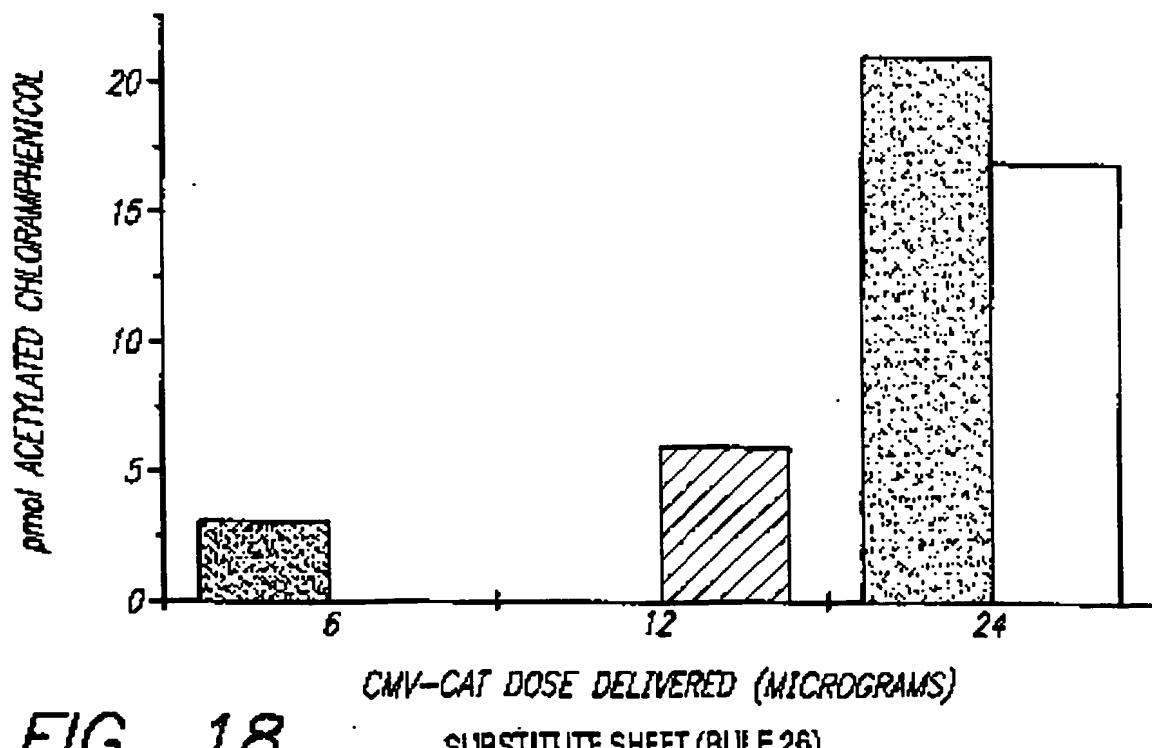
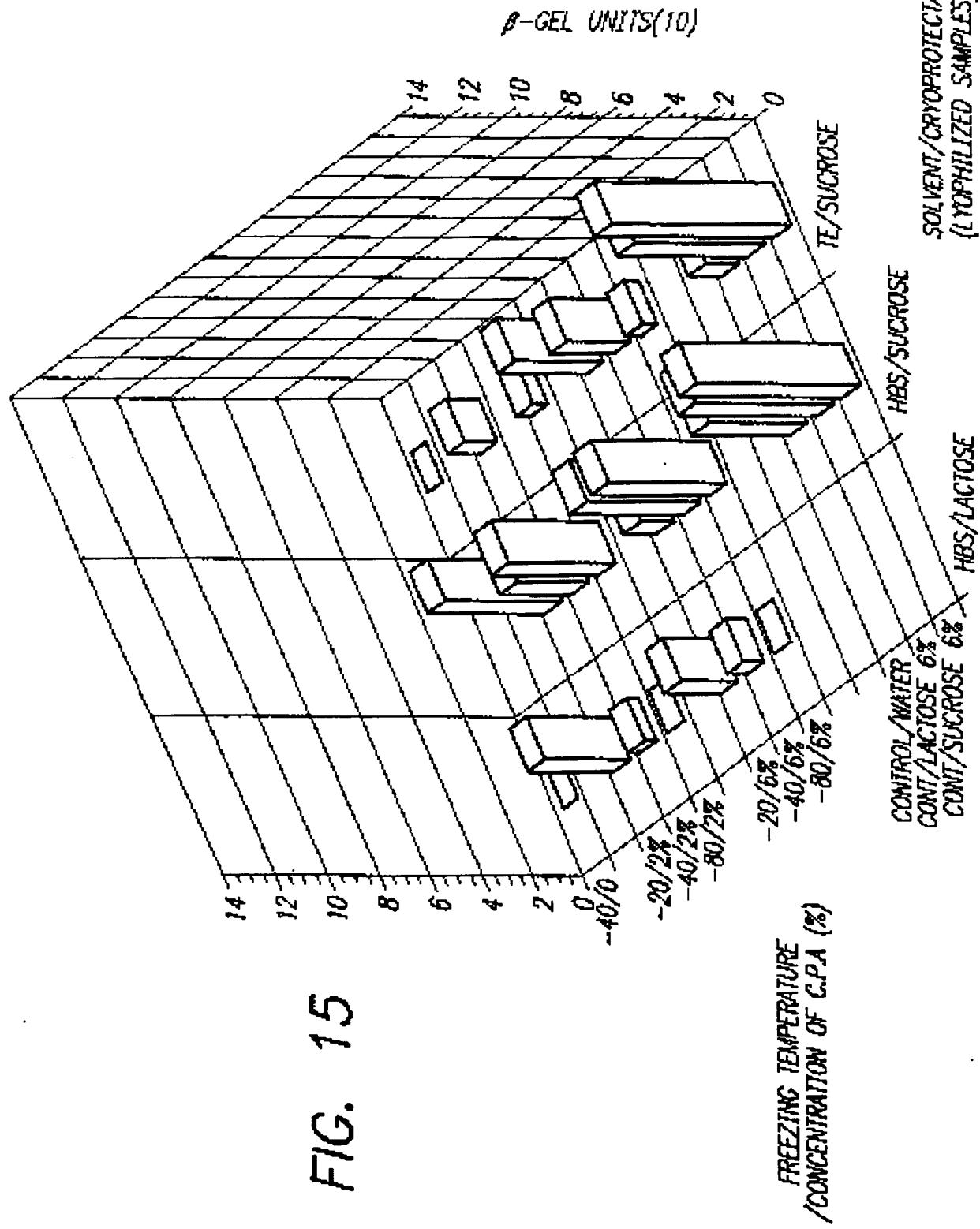


FIG. 18

SUBSTITUTE SHEET (RULE 26)

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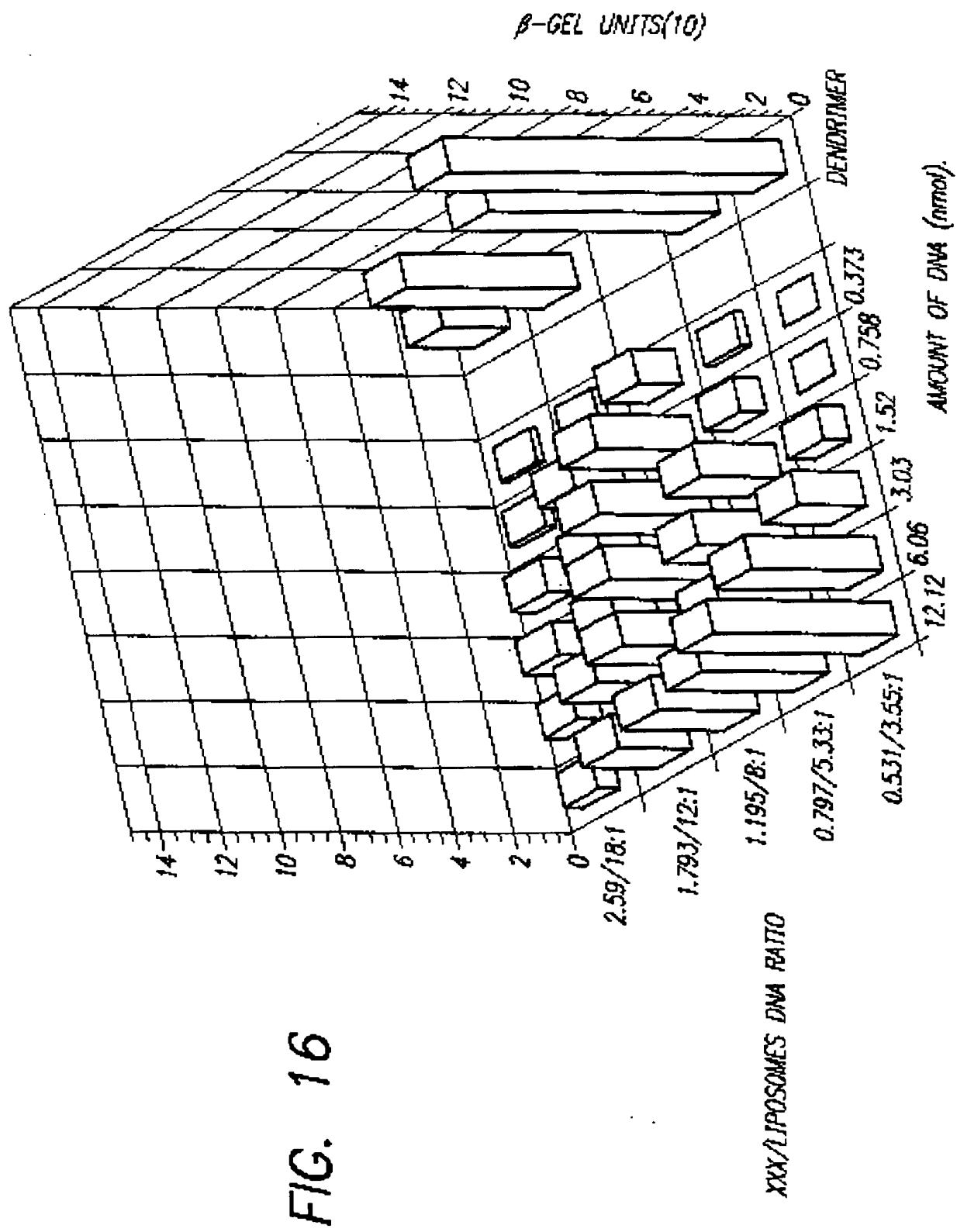
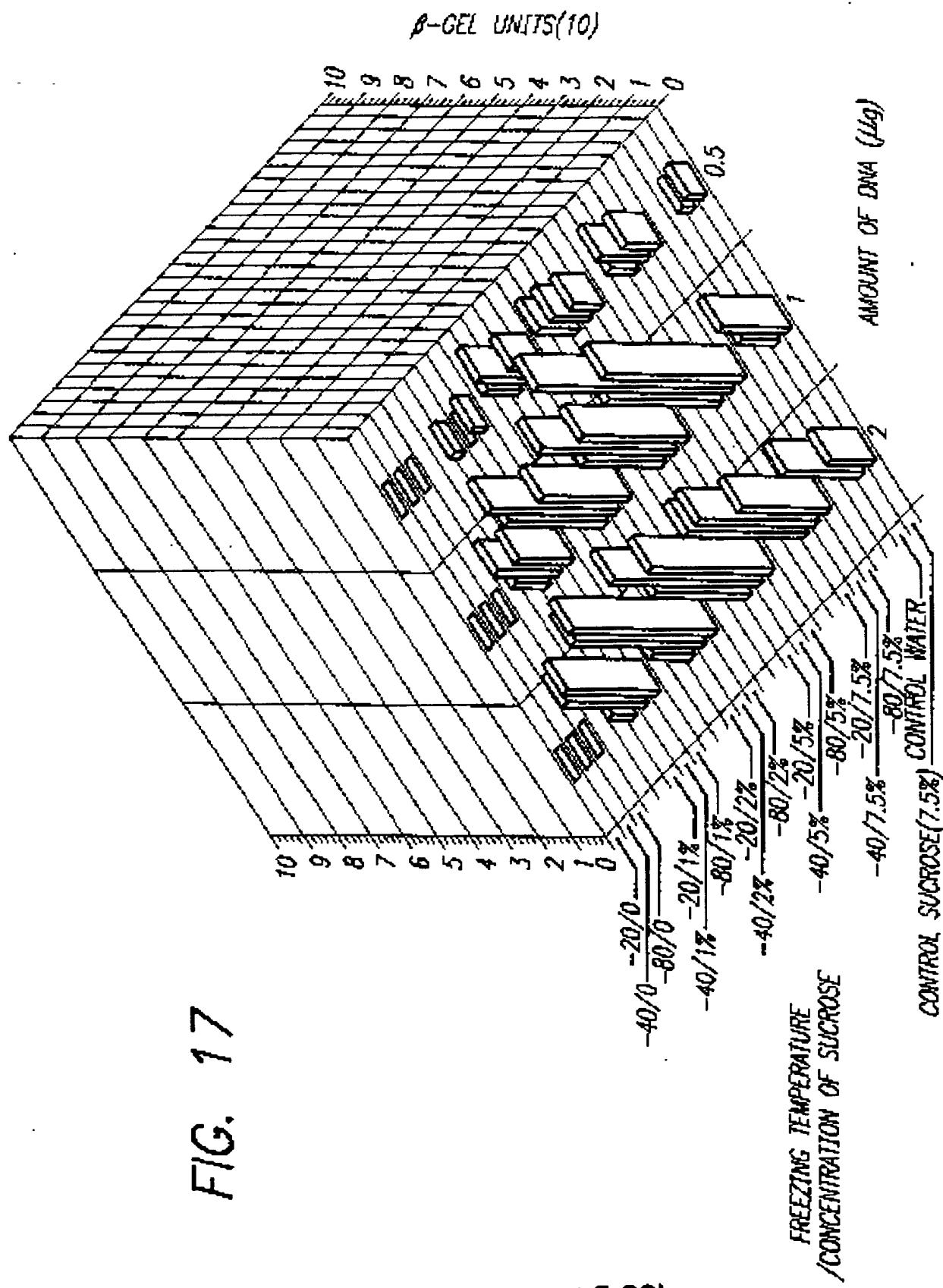


FIG. 16

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FIG. 19

SPERMINE-5-CARBOXYGLYCINE (*N'*-STEARYL - *N'*-OLEYL)  
AMIDE TETRATRIFLUOROACETIC ACID SALT (JK-75)

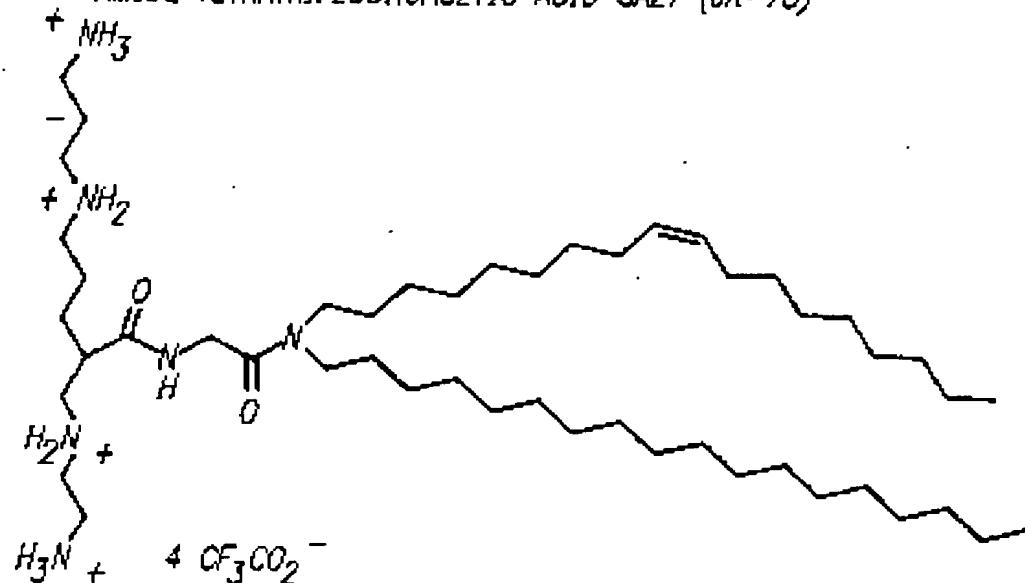
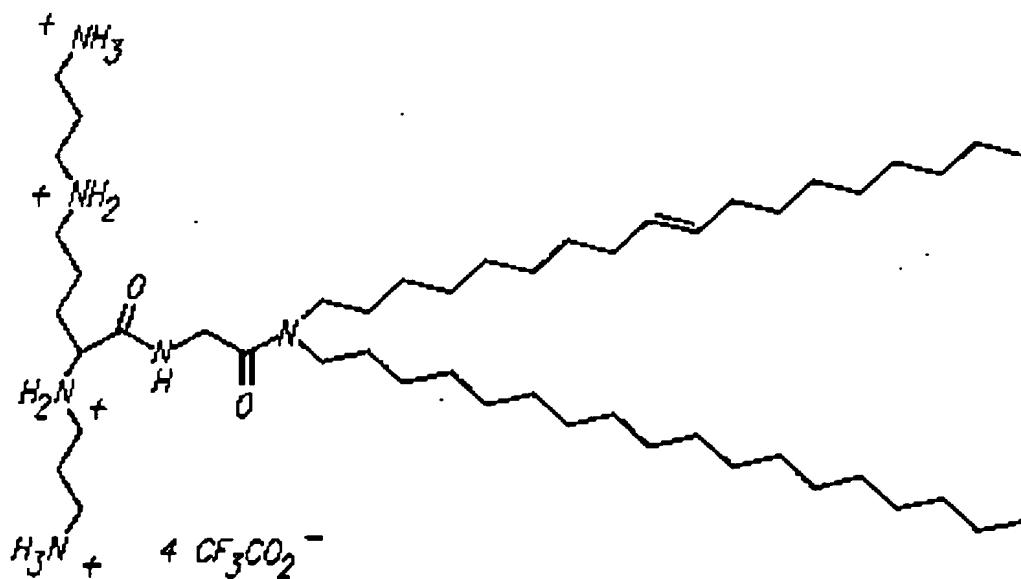


FIG. 20

SPERMINE-5-CARBOXYGLYCINE (*N'*-STEARYL - *N'*-ELAIDYL)  
AMIDE TETRATRIFLUOROACETIC ACID SALT (JK-76)



SUBSTITUTE SHEET (RULE 26)

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## AGMATINYL CARBOXYCHOLESTEROL ACETIC ACID SALT (AG-Chol)

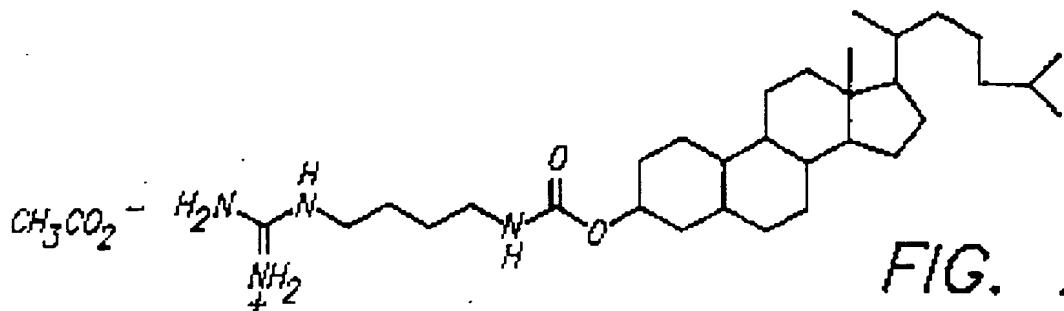


FIG. 21

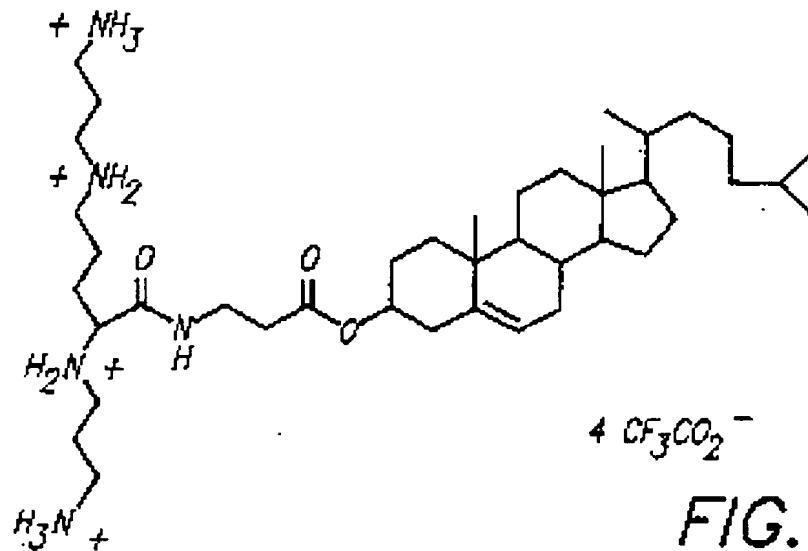
SPERMINE-5-CARBOXY- $\beta$ -ALANINE CHOLESTERYL ESTER  
TETRAFLUOROACETIC ACID SALT (CAS)

FIG. 22

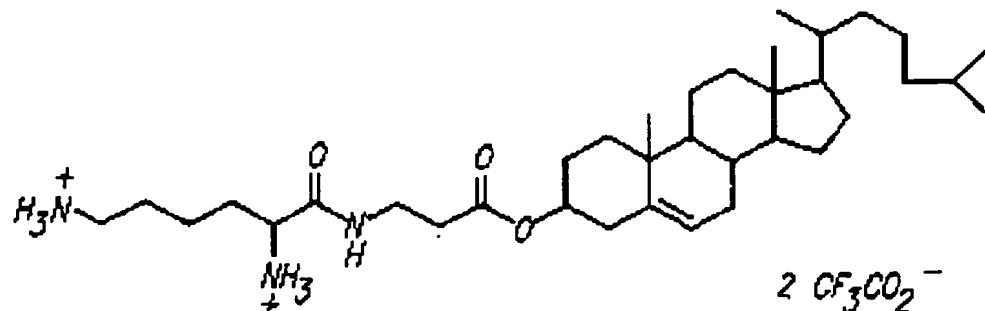
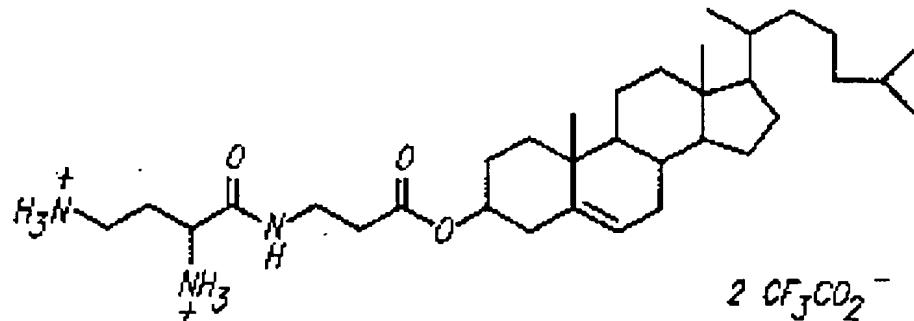
2,6-DIAMINOHEXANOYL  $\beta$ -ALANINE CHOLESTERYL ESTER  
BISFLUOROACETIC ACID SALT (CAL)

FIG. 23

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FIG. 24

2,4-DIAMINOBUTYROYL  $\beta$ -ALANINE CHOLESTERYL ESTER  
BISTRIFLUOROACETIC ACID SALT (CASB)



*N,N*-BIS (3-AMINOPROPYL)-3-AMINOPROPIONYL  $\beta$ -ALANINE CHOLESTERYL ESTER  
TRISTRIFLUOROACETIC ACID SALT (CASD)

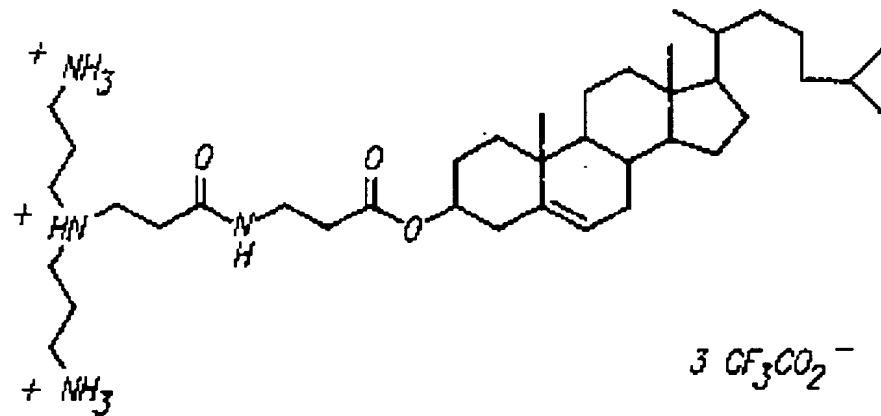


FIG. 25

[*N,N*-Bis(2-HYDROXYETHYL)-2-AMINOETHYL]AMINOCARBOXY CHOLESTERYL ESTER  
(JK-154)

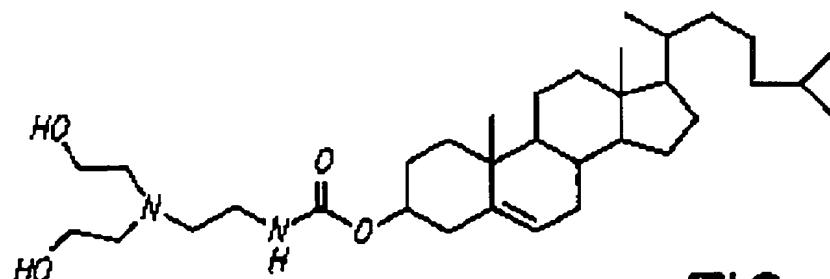


FIG. 26

## CARNITINE ESTER LIPIDS

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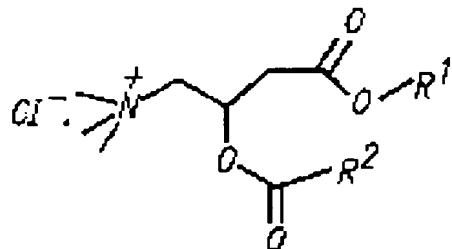
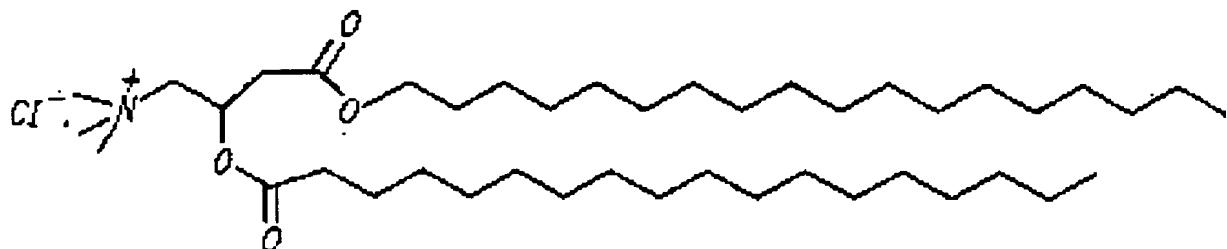


FIG. 27

$R^1 = (CH_2)_n CH_3$   $n = 2$  TO  $30$ ,  $1$  TO  $6$  UNSATURATED BONDS OR ISO  $CH_3$  GROUPS

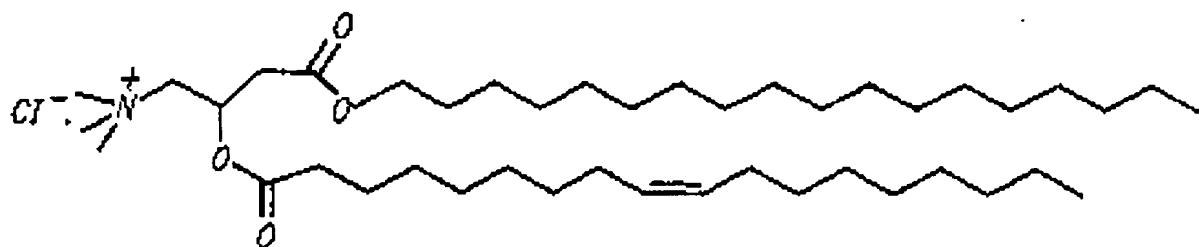
## STEARYL STEAROYL CARNITINE ESTER CHLORIDE SALT (SSCE)

FIG. 28



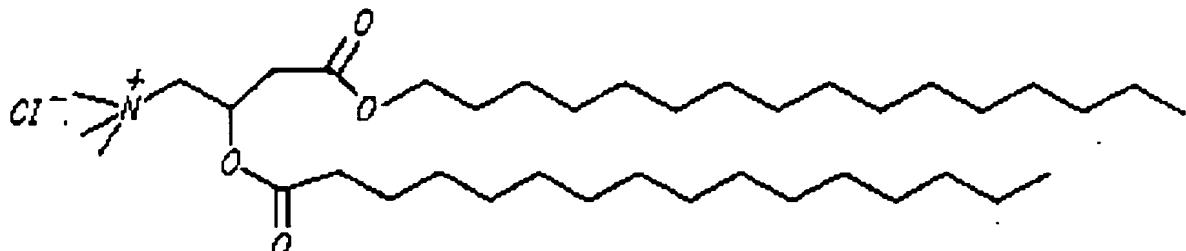
## STEARYL OLEOYL CARNITINE ESTER CHLORIDE (SOOC)

FIG. 29



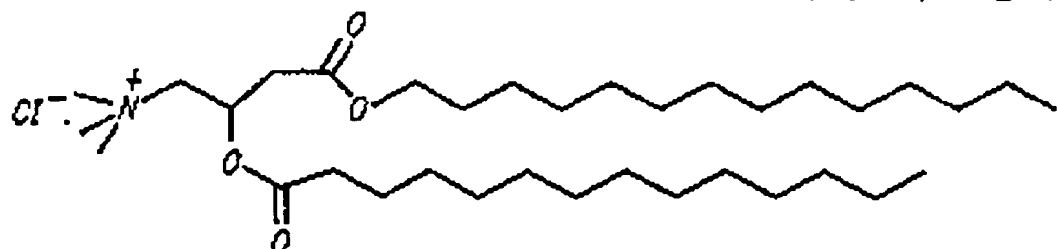
## PALMITYL PALMITOYL CARNITINE ESTER CHLORIDE (PPCE)

FIG. 30



## MYRISTYL MYRISTOYL CARNITINE ESTER CHLORIDE (MMCE)

FIG. 31



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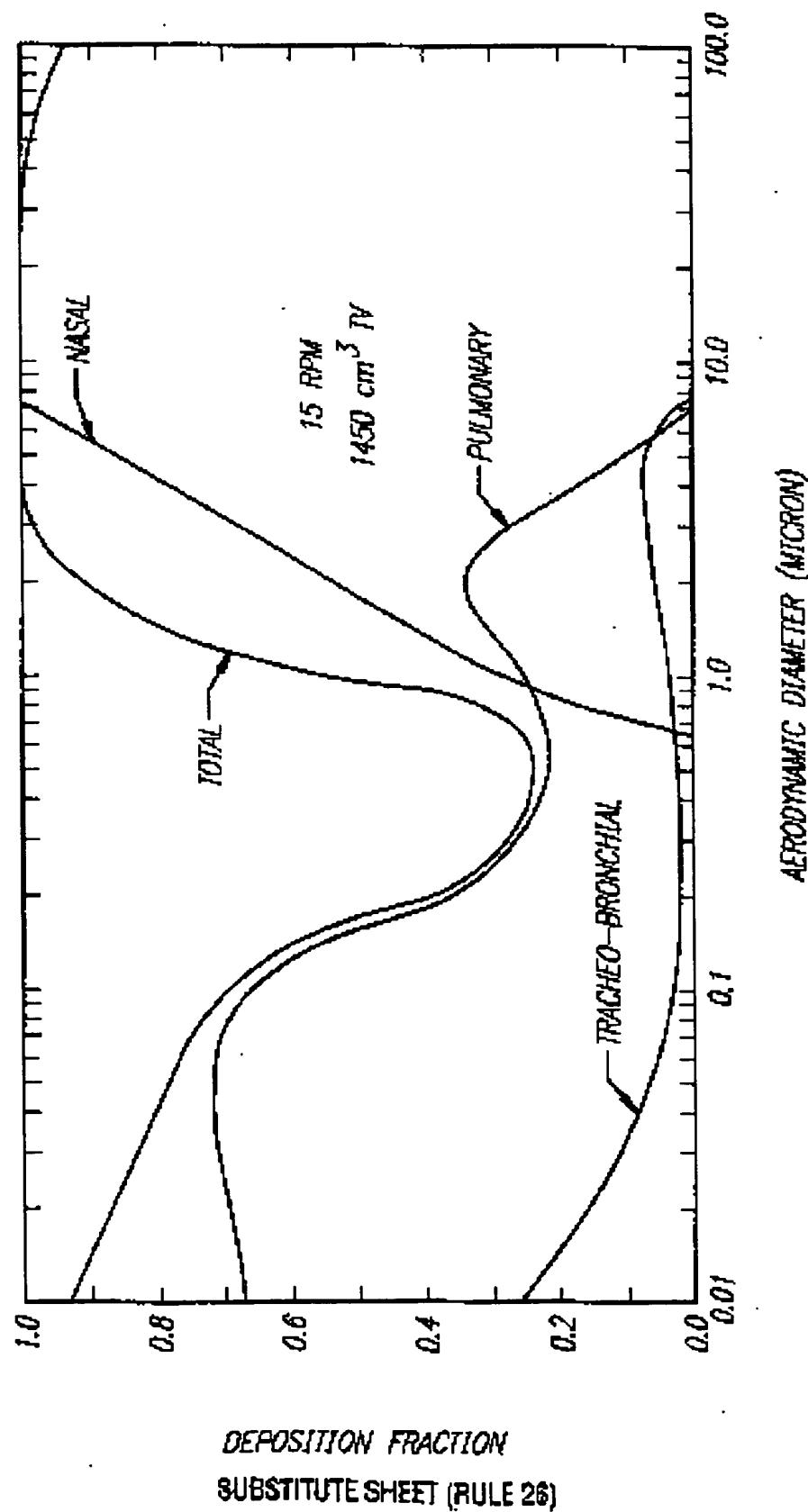


FIG. 32